XXXIII. THE FAT OF YEAST.

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Probably owing to the small quantity of material usually available, only one previous examination of yeast fat, that by Hinsberg and Roos [1903], appears to have been carried out, and, as this investigation was inconclusive on several points, it appeared to be worth while to go over the ground again. In an examination of yeast products for the "vitamines" or "hormones" connected with the phenomenon of animal growth, fairly large quantities of the crude fat of yeast became available and on this the following investigation was carried out. The results confirm the statement of the above mentioned workers that the principal saturated fatty acid present is a pentadecoic acid of empirical formula $C_{15}H_{30}O_2$, but in addition the author separated a small quantity of arachidic acid, $C_{20}H_{40}O_2$. There might be noted here the diversity of opinion in the literature as to the existence of a pentadecoic acid in the natural fats and the widely different values given to the physical constants of this acid by those authors who claim to have isolated it. The values given for the melting point vary from 70° to 52°, while the same constant for the methyl ester varies from 68° to 38°. Probably some of the acids described were impure as it is unlikely that a sufficient number of branched chain acids occur in the natural fats to account for the varying values given. The highest melting point obtained by the author for this acid was 59° while Hinsberg and Roos give 57°. An important point to be noticed is that for the synthetic normal acid of this composition, synthesised by Krafft [1879] and also by Le Sueur [1905], the melting point given is in both cases lower than the one found for the yeast acid, being stated as 51° by the former and 53° by the latter worker. The melting points of the simpler derivatives also disagree with those of the corresponding derivatives of the synthetic acid. The work was started in the belief that the substance was probably a mixture, but all attempts to separate it into more than one substance were unavailing and the only

conclusion possible was that the substance was really a single acid of the composition $C_{15}H_{30}O_2$ and, at the same time, that it was not the normal pentadecoic acid.

In dealing with the unsaturated acids the author experienced considerable difficulty in directly isolating any pure product, the simpler derivatives being difficult to deal with and indicating no satisfactory means of separation. By oxidation with potassium permanganate, however, very satisfactory specimens of the corresponding di- and tetra-hydroxy acids were obtained and these showed the presence in the original fat of the acids $C_{16}H_{30}O_2$, $C_{18}H_{34}O_2$ and $C_{18}H_{32}O_2$. Hinsberg and Roos obtained results which they interpreted as showing the presence of the acids $C_{12}H_{22}O_2$ and $C_{18}H_{34}O_2$ though they themselves query these formulae.

The phytosterol obtained melted at 145–147° and was apparently identical with that obtained by the other workers from one sample of beer yeast. The compound melting at 159° obtained by them from the majority of yeast samples appeared not to be present in the sample under examination.

Preliminary Examination.

The crude fat was hydrolysed with alcoholic potash and after the alcohol had been distilled off the crude dry soap was extracted with ether. From the ethereal solution the yeast cholesterol was obtained. The crude soap was then redissolved in water and after neutralisation of excess of alkali, the fatty acids were precipitated in the form of their lead salts and the usual rough separation of the salts of the saturated and unsaturated acids made by extraction with ether. The free acids were then liberated from their lead salts by hydrochloric acid and treated as described below.

The Saturated Fatty Acids.

When the dark brown solid mass, obtained from the ether-insoluble lead salts, was distilled under a pressure of 100 mm. by far the larger part passed over between 250° and 260°. This was redistilled and came over at about 215° under 15 mm. pressure. In the first distillation the process was not pressed to an extreme extent and the residue left in the flask was purified by crystallisation only. The portion which had been twice distilled was crystallised several times from methyl alcohol and then melted at 54–55°. An iodine absorption determination gave however an iodine value of 29·5, and, as this was only very slowly altered by crystallisation, the acid was dissolved in dilute potash solution and treated with 1·5 per cent. permanganate

solution so as to convert the impurities consisting of unsaturated acids into the corresponding hydroxy-acids. The solution having been treated with sulphur dioxide, the precipitated acids were filtered off and treated with a small quantity of ether. Filtration from the hydroxy-acids and crystallisation of the substance obtained from the ethereal solution gave a white crystalline body, melting at 59° and showing no iodine absorption when treated with Hübl's solution.

Pentadecoic Acid.

On analysis the substance, whose preparation has just been described, gave the following result:

0·1686 g.; 0·4611 CO_2; 0·1878 H_2O.
Found 74·59 % C; 12·38 % H.
Calc. for $C_{15}H_{30}O_2$ 74·38 % C; 12·39 % H.

This substance was evidently the same as that separated by Hinsberg and Roos and described by them as being definitely pentadecoic acid.

A determination of the molecular weight by the freezing point method showed that 0·4310 g. substance dissolved in 24·2174 g. glacial acetic acid gave a depression of the freezing point of 0·295°; whence molecular weight = 235. Calculated for $C_{15}H_{30}O_2 = 242$. On titration with alcoholic potash 1·8344 g. acid took 14·9 c.c. N/2 KOH for neutralisation.

KOH required = 22·74 % weight of acid.
Calc. for $C_{15}H_{30}O_2$ = 23·14 % weight of acid.

The silver salt, prepared by precipitating a neutral solution of the potassium salt with silver nitrate, was obtained as a white amorphous precipitate and gave the following result on ignition:

0·4288 g. salt; 0·1308 g. metallic silver. Molecular weight of the acid = 247.
Calc. for $C_{15}H_{30}O_2$ = 242.

The above estimations point to the substance being pentadecoic acid or a mixture with practically the same composition. If not a single compound the substance was most likely to be a mixture of two of the three acids, myristic, palmitic and stearic acid. Referring to the determinations by Heintz of the melting points of mixtures of any two of these acids it was found that with a melting point of 59° the substance might be a mixture of (*a*) 15 % myristic with 85 % palmitic acid, (*b*) 40 % myristic with 60 % stearic acid, (*c*) 45 % palmitic with 55 % stearic acid. Assuming the substance to be one of these mixtures, weighed quantities of pure myristic, palmitic or stearic acids were added and the melting point curves plotted. In no case did the curve agree with that for any pair of the above three acids. The melting point for a

mixture of all three of the above acids lies below that actually determined. Other methods for the separation of a possible mixture in the substance were tried but without result. Thus the fractional precipitation of the magnesium salts and the continual crystallisation of the lithium salts from alcohol were without effect. To obtain further evidence of the homogeneous nature of the substance several derivatives were prepared and compared with the corresponding compounds of the better known fatty acids.

The *methyl ester*, prepared by passing gaseous hydrochloric acid into a mixture of the acid and methyl alcohol, was obtained as a white crystalline substance which could be crystallised from a small quantity of methyl alcohol and melted at 26°, which is considerably lower than the melting point for either methyl palmitate or methyl stearate.

The *anilide* was prepared by boiling the acid for some hours with excess of aniline. It crystallised from alcohol in pearly scales and melted sharply at 86–87°.

Myristic anilide melts at 84°, palmitic anilide at 90·5° and stearic anilide at 94°.

Analysis: 0·2117 g.; 10·3 cc. moist N at 16° and 772 mm. N = 4·48 %.
Calc. for $C_{21}H_{35}ON$ N = 4·41 %.

The *amide* was prepared by heating together equal quantities of phosphorus pentachloride and the acid in chloroform. The solvent and phosphorus oxychloride were distilled off under slightly reduced pressure and the residue poured into strong ammonia. It crystallised in small flakes from 50 % alcohol and melted at 94–95°.

The amide of the synthetic pentadecoic acid, prepared by Le Sueur, melted at 102·5°, while palmitic amide melts at 107°.

0·1443 g.; 7·0 cc. moist N at 15° and 760 mm. N = 5·71 %.
Calc. for $C_{15}H_{31}ON$ N = 5·81 %.

The whole of the evidence points to the substance being a definite compound of the composition $C_{15}H_{30}O_2$, but at the same time being not identical with the normal synthetic acid of this composition.

Arachidic Acid.

It has been mentioned that a small quantity of material remained behind in the flask when the pentadecoic acid was distilled under reduced pressure. This small quantity of material was several times crystallised from alcohol and was finally obtained as a white crystalline body which melted sharply at 77°.

On analysis it gave the following results:

0·1212 g.; 0·3440 g. CO_2; 0·1385 g. H_2O.
C=77·40 %, H=12·70 %.
Calc. for $C_{20}H_{40}O_2$ C=76·92 %, H=12·82 %.
0·4585 g. required 14·2 cc. N/10 KOH for neutralisation.
KOH required=17·34 % of weight of acid.
Calc. for $C_{20}H_{40}O_2$ 17·95 % of weight of acid.

The substance was optically inactive, did not absorb iodine from Hübl's solution and did not react with acetic anhydride. The substance was almost certainly therefore arachidic acid.

The Unsaturated Acids.

The unsaturated fatty acids, liberated from the ether-soluble lead salts, formed a dark brown oily mass which behaved as a "semi-drying" oil when exposed in films to the air. Attempts were at once made to purify this mass by distillation under reduced pressure but without much success, as decomposition of some portion took place and the distillation was rendered useless. Distillation in steam was also of little avail the quantity passing over being very small. In an attempt to obtain some separation by means of salts, it was noticed that the barium salts, a sticky mass containing a small quantity of water, were soluble to some extent in benzene, toluene and ether, but almost insoluble in cold alcohol while with hot alcohol the mass yielded an apparently homogeneous glue with the solvent. These observations together with an iodine value of 138 for that portion of the acids whose barium salt was soluble in benzene, point to the presence in the mass of some acids of the linoleic series and the presence of some proportion of these acids would account for the "semi-drying" properties of the oil.

Attempts at direct separation of the mixture having failed, the whole "unsaturated" fraction was neutralised with caustic potash, dissolved in a large volume of water and an equal volume of 1·5 per cent. potassium permanganate solution run into it. After standing for ten minutes sulphurous acid solution was added and the precipitated acids filtered off. The filtrate was found not to contain any appreciable quantity of hydroxy-acids, thus showing the absence in the original mixture of any appreciable quantity of acids of the linoleic series.

The acids precipitated by sulphur dioxide were washed with small quantities of ether to remove unoxidised material and afterwards treated with large quantities of the same solvent. The ether-soluble portion was crystallised from alcohol and the purified dihydroxy-acid is described below. The portion insoluble in ether was crystallised from large quantities of water and was identified in the manner hereafter described as a tetrahydroxy-acid.

The Dihydroxy-acid.

This substance was obtained as a well crystallised white powder, insoluble in water, slightly soluble in ether but soluble in hot alcohol. It melts at 124–125°.

Analysis: 0·1568 g.; 0·3894 g. CO_2; 0·1566 g. H_2O.
C = 67·73 %, H = 11·09 %.
Calc. for $C_{17}H_{32}O_2(OH)_2$ C = 67·55 %, H = 11·25 %.
0·6453 g. required 21·0 cc. N/10 KOH for neutralisation.
KOH = 18·22 % of weight of acid.
Calc. for $C_{17}H_{32}O_2(OH)_2$, KOH = 18·54 % of weight of acid.

The acetyl derivative gave the following result:

0·8212 g. gave acetic acid equivalent to 53·7 cc. N/10 acetic acid.
Acetic acid = 40·45 % weight of substance taken.
Calc. for $C_{17}H_{34}O_4$ 39·73 %.

The above figures point to the substance being a dihydroxy-acid of the composition $C_{17}H_{34}O_4$ and the melting point and general properties agree exactly with a substance of this composition described by Fahrion [1893]. This substance was shown by Ljubarsky [1898] to be probably a mixture of dihydroxypalmitic acid and dihydroxystearic acid, which mixture the latter author found to behave in many respects as a chemical individual. In view of the fact that an unsaturated acid of the oleic series with the composition $C_{17}H_{32}O_2$ has hitherto not been separated with certainty from natural fats, it seems more probable that the substance separated was the mixture which Ljubarsky claims it to be, than a single chemical individual, although no separation was possible by repeated crystallisation from alcohol.

If this is so the original fat must have contained two unsaturated fatty acids of the oleic series with compositions $C_{16}H_{30}O_2$ and $C_{18}H_{34}O_2$ respectively.

Tetrahydroxy-acid.

The oxidation product insoluble in ether but crystallising from large quantities of water was analysed with the following results:

0·1465 g.; 0·3330 g. CO_2; 0·1380 g. H_2O.
C = 61·99 %, H = 10·46 %.
Calc. for $C_{18}H_{32}O_2(OH)_4$ C = 62·07 %, H = 10·34 %.
1·0878 g. requires 32·3 cc. N/10 KOH for neutralisation.
KOH = 16·63 % of acid.
Calc. for $C_{18}H_{32}O_2(OH)_4$ KOH = 16·18 %.
0·4321 g. when converted into acetyl compound and hydrolysed gave acetic acid equivalent to 50·5 cc. N/10 acetic acid.
Acetic acid obtained = 70·12 % of weight of acid taken.
Calc. for $C_{18}H_{32}O_2(OH)_4$ 68·96 %.

The substance is a white crystalline body of silky lustre, melting at 156°, soluble in hot alcohol and glacial acetic acid but insoluble in nearly all other solvents. The general properties of the acid, together with the analyses given above, point to the substance being a tetrahydroxy-acid of the composition $C_{17}H_{31}(OH)_4 . COOH$. The best known acid of this composition is tetrahydroxystearic acid (sativic acid), formed by the oxidation of linoleic acid, but this melts at 173–174°. Apparently, however, there are several isomeric acids of this composition, for other observers have reported acids melting at 152° and 165° and the acid separated may be identical with one of these. At any rate, the isolation of this substance points definitely to the existence in yeast fat of an unsaturated acid of the linoleic series with the empirical composition $C_{18}H_{32}O_2$, that is, an acid certainly isomeric, but perhaps not identical with ordinary linoleic acid.

Yeast Cholesterol.

The ethereal extract of the crude potassium salts obtained from the first hydrolysis of the yeast fat was worked up in the usual way for the yeast cholesterol. The product obtained, after being crystallised several times from alcohol, melted at 145–147°, and this melting point was not altered by repeated crystallisation. It had $[\alpha]_D = -75{\cdot}54°$ and gave similar colour reactions to the majority of the phytosterols. It was apparently identical with the cholesterol obtained from one sample of yeast by Hinsberg and Roos. The cholesterol, melting at 159°, which these authors most frequently obtained from yeast was not isolated in spite of a careful search being made for it.

Summary.

A comparison of the results obtained by Hinsberg and Roos and by the author is shown in columns I and II respectively of the following table.

I	II
Saturated acid $C_{15}H_{30}O_2$. M.P. 56°.	Saturated acid $C_{15}H_{30}O_2$. M.P. 59°.
Not identified in the mixture.	Saturated acid $C_{20}H_{40}O_2$. M.P. 77°. Arachidic Acid.
Unsaturated acid $C_{12}H_{22}O_2$.	Not identified in the mixture.
Unsaturated acid $C_{18}H_{34}O_2$.	Unsaturated acids $C_{16}H_{30}O_4$ and $C_{18}H_{34}O_2$. Presence shown by their oxidation products.
Not identified in the mixture.	Unsaturated acid $C_{18}H_{32}O_2$, shown by its oxidation product.
Yeast cholesterols melting at 159 and 145–148°.	Yeast cholesterol melting at 145–147°.

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XXXIV. THE INFLUENCE OF THE CARBONATES OF THE RARE EARTHS (CERIUM, LANTHANUM, YTTRIUM) ON GROWTH AND CELL-DIVISION IN HYACINTHS.

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The effects of various organic and inorganic bodies on growth and cell-division in both plants and animals have been studied by several researchers in this laboratory during the past few years. Moore, Roaf, and Whitley [1905] investigated the action of acids and alkalis on the development of the fertilized eggs of the sea-urchin, *Echinus esculentus*. Their inquiry was directed to the effects of variation in hydrogen and hydroxyl ion concentration in the medium (sea-water) in which the eggs were allowed to develop, and they found that within certain narrow limits of concentration, alkalis favoured cell-division, whereas acids were invariably fatal. Small additions of acids inhibited cell-division and growth, and at a concentration of 0·001 molar practically all cell-division was stopped. A slight increase in alkalinity favoured development, at the same time producing irregularities in cell-division. Beyond the optimum concentration of alkali, exceedingly irregular division, resulting in particular in the production of multi-nucleated cells, was observed. At a concentration of 0·0015 M. of caustic alkali, however, cell-division was completely arrested.

Whitley [1905] observed similar effects on the eggs of plaice, and also noted the action of the indicators phenolphthalëin and dimethylaminoazobenzene on the eggs both of plaice and echinus. He found that while phenolphthalëin was deadly to the eggs of echinus, it was harmless to those of the plaice. On the other hand, the azobenzene derivative quickly killed the latter, and appeared, if anything, to have a favourable effect on the development of the former.

Moore, Knowles, and Roaf [1908] extended the observations to plants, using for their experiments the common hyacinth. They obtained similar

results in regard to the influence of acids and alkalis, and they also showed that the cation had a specific effect, potassium being much more stimulating than sodium to both rootlets and foliage leaves. The phosphatic ion also had a special effect on the flower, causing an increase in size at optimum concentration, and at higher concentrations an irregular inflorescence, with packed florets on a dwarfed stalk.

Histologically they observed depression of nuclear division with acids, and thickening of the cell walls; with alkalis, increased nuclear division, changes in chromosomes and irregular figures, while the cell outlines became obscured.

Coppin [1912] studied the effect of allantoin and other purine derivatives on the growth of hyacinths, and also salts of organic acids such as sodium huminate, sodium malate, and sodium oxalate.

These latter substances, as well as sodium urate, had a stimulating effect on the growth of the hyacinths, but allantoin and the other purine substances inhibited both growth and cell-division.

Working on somewhat different lines, Ransom [1912] observed the action of caffeine upon the germination of seeds. He used a large number of different seeds, and his method consisted in soaking the seeds for a short time in his caffeine solutions before sowing them in the usual manner. He found that caffeine, even in a very dilute solution, had a powerful effect in retarding germination and growth; while a concentration of 1 per cent. in many cases completely inhibited germination.

In the present inquiry, salts of the rare earths were used to test whether these produced any physiological effects on growth and cell-division. For this purpose the carbonates of cerium, lanthanum, and yttrium were selected.

Preparation of carbonates.

The carbonates were prepared, according to Moissan, in the following ways:

(1) Cerium. A solution of cerium nitrate was treated with ammonium carbonate, and the precipitate of cerium carbonate filtered off, and thoroughly washed.

(2) Lanthanum and Yttrium. The hydroxides of the metals were taken, suspended in water, and thoroughly saturated with carbon dioxide over a period of several hours. The precipitates were then filtered off.

Preparation of the carbonate solutions.

Owing to the very slight solubilities of the carbonates of cerium, lanthanum and yttrium, some difficulty was experienced in preparing solutions. Finally two or three grams of each of the respective carbonates were suspended in about two litres of Liverpool tap-water[1], and carbon dioxide was passed into the bottles for one or two hours, thus ensuring complete saturation. After being allowed to stand overnight, the undissolved residues were filtered off.

These clear filtrates were the actual solutions used in the experiments. Their concentrations were estimated by taking a measured volume of each, evaporating to dryness, and weighing the residue. The following figures were obtained:

Cerium	0·007 %
Lanthanum	0·01 %
Yttrium	0·017 %

In all probability they contained a mixture of the respective carbonates and bicarbonates.

Effect on the growth of hyacinths.

The plants used were a common variety of hyacinth. Healthy bulbs of as nearly uniform size as possible were selected. They were placed in hyacinth glasses which had been blackened on the outside with black lacquer to prevent action of light upon the rootlets. The hyacinth glasses held about 450 cc. and the solutions mentioned were filled in until they just wetted the bulbs. In addition, controls were grown under precisely similar conditions in Liverpool tap-water. Water was added to the glasses from time to time to make up for the loss due to evaporation. A few of the ends of the growing rootlets were cut off on the twenty-fifth day for the purpose of studying the effects of the solutions on cell-division and nuclear changes. These were all immediately fixed in Flemming's solution, cut in paraffin, and stained for nuclear figures by Heidenhain's iron-alum, haematoxylin method.

On the twenty-fourth day, a measured volume of the fluid was removed from each of the different solutions, filtered, and evaporated to dryness, organic matter being removed by ashing with ammonium nitrate. The residues were then weighed. Results:

[1] Liverpool tap-water is very pure surface water and practically free from inorganic salts.

	24th day	Original
Cerium	0·0068 %	0·007 %
Lanthanum	0·0076 %	0·01 %
Yttrium	0·0066 %	0·017 %

Thus in the case of the cerium carbonates there had been little or no absorption; but in the other two cases, particularly in that of yttrium, there had been marked absorption.

In addition measurements and observations were made at intervals of the growth and conditions of the plants, the points noted being the length of the green leaves, length and condition of the rootlets, and the condition of the florets and the flower spike. These observations and measurements will be found set out in the accompanying Table (p. 353).

While the results obtained were not in all cases concordant, a few definite points may be made. In the first place, all the bulbs in the experimental solutions reached maturity before the controls in water.

Secondly, the plants in the cerium carbonate solution were the first to attain maturity, though their development was not so marked as that of those in the lanthanum carbonate, which followed next in point of time.

The effect of the yttrium solution was somewhat anomalous, since while all the plants matured a few days before the controls, the rootlets were dwarfed, and looked yellow and unhealthy from the beginning.

The lanthanum ion seems to have a special effect on the flower stalks, resulting in very tall plants. No irregularities manifested themselves in the inflorescences.

Histological investigation of growing root-tips under the influence of the above reagents.

The varying effects produced by the different metallic ions are best seen from the accompanying photomicrographs (Plate I). Speaking generally it may be stated that the cerium and lanthanum ions have a decidedly stimulating effect on cell-division in the rootlets, while that of yttrium has a deleterious effect.

The following are brief notes of the histological examination of slides prepared from each of the twelve plants as mentioned in the Table. They were stained by Heidenhain's iron-alum, haematoxylin method.

1. *Cerium carbonate.* (*Nos.* 2 *and* 3.) (*See photomicrograph* 2.)

In both these preparations a noticeable feature is the beautiful regularity of the arrangement of the cells, the sections showing a large number of

Measurement of roots and leaves in cm.

Started Dec. 16th		Jan. 8, 23rd day Roots only	Feb. 8, 54th day		Feb. 11, 57th day		Feb. 15, 61st day		Feb. 24, 70th day		Feb. 28, 74th day		Mar. 4, 78th day	
			Roots	Leaves	Roots	Leaves	Roots	Leaves	Roots	Leaves	Roots	Leaves	Roots	Leaves
Cerium Carbonate 0·007 %	1	1	1 Look rotten	4·5 Withered	No development									
	2	1	1	7	1	7 All florets open	—	—	—	—	—	—	—	—
	3	7·5	12	12	15	15	15	17·5 All florets open	—	—	—	—	—	—
Lanthanum Carbonate 0·01 %	4	12·5	15	10	15	17	15	17 All florets open	—	—	—	—	—	—
	5	7	7·5	10	8	12·5	8	15 Florets open	—	—	—	—	—	—
	6	4	4	8	5	10	5	12·5	5	12·5 Florets open	—	—	—	—
Yttrium Carbonate 0·017 %	7	2·5	4	7·5	4	10	6	12·5 Florets open	—	—	—	—	—	—
	8	2	2	7·5	2	12·5	2	12·5	2	12·5	2	15 Florets open	—	—
	9	2	2·5	6	3	7·5	3	7·5	3	7·5	3	8 Florets open	—	—
Controls Water	10	7	10	10	10	12·5	10	12·5	10	15	10	15	12	16 Florets open
	11	4	6	10	6	11·5	6	12	7·5	12·5	7·5	15	7·5	15 Florets open
	12	2	4·5	7·5	5	10	5	10	7·5	12·5	Withered			

rather small, closely packed, square cells, containing many dividing nuclei. The cell-walls are clearly defined, and the cytoplasm is granular and stains well. In many cases the nuclei are elongated, and show one or two nucleolus-like chromatin dots surrounded by a clear space.

2. *Lanthanum carbonate.* (*Nos.* 4, 5 *and* 6.) (*See photomicrograph* 3.)

The same regularity of the arrangement of the cells is seen in these sections, but it is not quite as marked as in the cerium preparations. Many dividing nuclei are seen, and the nucleolus-like dots above referred to are also conspicuous. The cytoplasm is faintly granular, and the cell walls are less sharply defined than in Nos. 2 and 3 (cerium).

3. *Yttrium carbonate.* (*Nos.* 7, 8 *and* 9.) (*See photomicrograph* 4.)

In this series hardly any dividing cells are to be seen. The arrangement of the cells is irregular. The nuclei are very deeply stained, and irregular in size and shape. The cell walls are not distinct, and the cytoplasm is scanty, ill-defined, and very faintly staining. The whole appearance is that of an irregular mass of cells, with scattered deeply-stained nuclei, and presents a very different picture from the compact and regular arrangement shown in the cerium and lanthanum series.

4. *Controls—tap-water.* (*Nos.* 10, 11 *and* 12.) (*See photomicrograph* 1.)

In these sections no great amount of cell-division is noticeable. The cells are fairly regularly arranged, and the nuclei are deeply stained. They are for the most part in the resting condition, and many exhibit the darkly staining dots resembling nucleoli, each surrounded by a clear space.

Conclusions.

1. Marked effects are produced upon the dividing cells of hyacinth rootlets by the addition of the carbonates of cerium, lanthanum and yttrium to the medium. The concentration of these substances necessary to produce physiological effects is very small.

2. The cations produce diverse effects; lanthanum especially, and cerium being favourable to growth and cell-division, while yttrium is unfavourable.

3. The lanthanum ion has a special effect on the flower stalk, causing an increase in length.

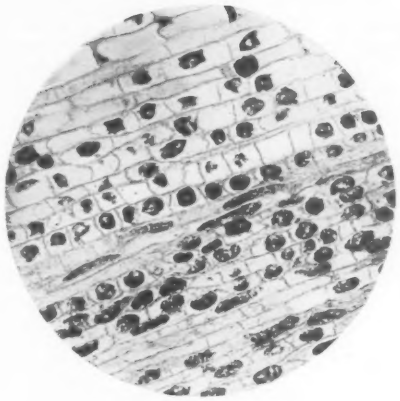

Fig. 1.

Control—tapwater. ×460 diameters.

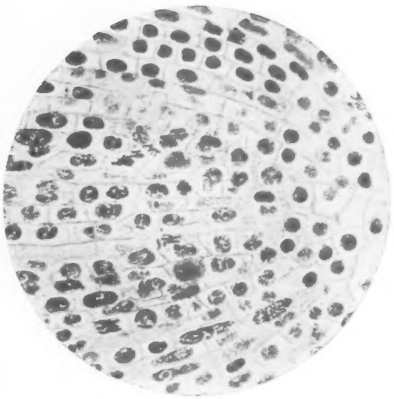

Fig. 2.

Cerium carbonate ·007 %. ×460 diameters.

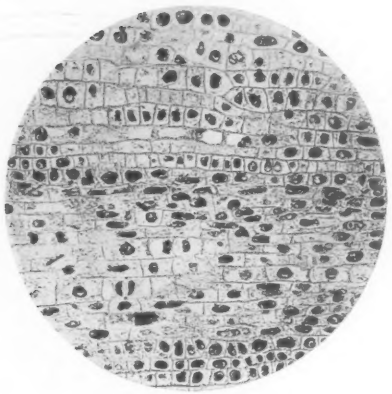

Fig. 3.

Lanthanum carbonate 0·01 %. ×460 diameters.

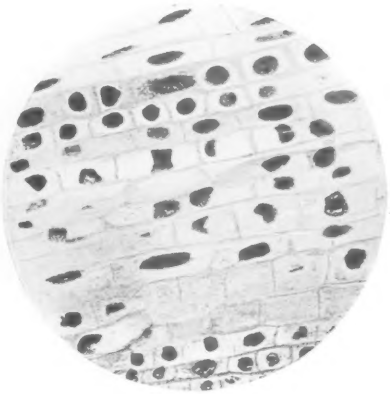

Fig. 4.

Yttrium carbonate ·017 %. ×460 diameters.

4. The cytological effects are best seen in the accompanying photographs. In the cerium and lanthanum preparations there is a marked increase in cell-division, accompanied by a beautiful regularity in the arrangement of the cells. With yttrium there is a diminution in cell-division, and the cells are irregularly arranged.

I should like to take this opportunity of thanking Professor Benjamin Moore for suggesting the line of research, and for his kindly help and criticism throughout the work.

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XXXV. ON THE CHEMICAL NATURE OF SUBSTANCES FROM ALCOHOLIC EXTRACTS OF VARIOUS FOODSTUFFS WHICH GIVE A COLOUR REACTION WITH PHOSPHOTUNGSTIC AND PHOSPHOMOLYBDIC ACIDS. (PRELIMINARY COMMUNICATION.)

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From the Biochemical Department, Lister Institute.

(*Received May 22nd, 1913.*)

Two colour reactions have recently been described, one with phosphotungstic acid for uric acid [Folin and Macallum, 1912], the other with phosphotungstic and phosphomolybdic acids for polyphenols [Folin and Denis, 1912], which they recommend for the determination of these substances. One of us previously observed during the fractionation of yeast and rice-polishings [Funk, 1913] that the vitamine-fraction constantly gave a blue coloration with both reagents, that the reaction gradually disappeared during the further purification of the products and at the same time that the curative power for polyneuritis in birds slightly diminished. As these reacting substances might be of some importance for the process of curing, we have tested a large number of compounds with both reagents, in order to obtain some information as to the chemical nature of these substances, and we have found that a certain number of them which occur in nature show the colour reactions.

With the uric acid reagent certain purine bodies and some tyrosine derivatives give a very strong reaction. The alcoholic extracts of different foodstuffs, especially those which are known to be rich in vitamines, give in general both reactions very markedly. The substances giving the reaction seem to pass into the phosphotungstic acid filtrate and we are proceeding to isolate them.

The reactions with purine and pyrimidine derivatives.

Substance	Uric acid reagent	Phenol reagent
Xanthine	–	++
Hypoxanthine	–	+
Paraxanthine	–	–
Guanine	–	++
Alloxantin	+++	+++
Alloxan	–	–
Thymine	–	–
Theophylline	–	–
3-Methyl-uric acid	+	++
7- „ „	trace	++
Hydantoin	–	–
Uracil	–	–
Guanidine carbonate	–	–
Hydantoic acid	–	–
Allantoin	–	–
Adenine	–	–
Uridine	–	–
Guanosine	–	trace
Adenosine	–	–
Cytidine nitrate	–	–
Yeast nucleic acid	trace	trace
Thymonucleic acid	–	–

Tyrosine derivatives.

l-Tyrosine	–	+++
Nitro-tyrosine	–	–
3–4-Dihydroxyphenylalanine	+++	+++
2-Aminotyrosine	+++	+++
3-Aminotyrosine	++	+++
l-Tyrosine anhydride	–	–?
Glycyl-*l* tyrosine	trace	++

We have also investigated a number of amino-acids, polypeptides and diketopiperazines, not including tryptophane, oxy-tryptophane and oxy-proline, all of which were entirely negative to both reagents.

Foodstuffs.

Ceridin (alcoholic extract of yeast)	trace	+
Zymin	trace	+
Alcoholic extract of rice-polishings	+	++
Subs. $C_{24}H_{19}O_9N_5$ from vitamine-fraction of yeast	–	–
Subst. $C_{29}H_{23}O_9N_5$ from the same fraction	–	+
Nicotinic acid from yeast and rice	–	–
$C_{26}H_{20}O_9N_4$ from vitamine-fraction of rice	–	–
Alcoholic extract from caseinogen (crude)	+	+
Alcoholic extract of milk	+	+
Whey powder from milk	+	+
Filtrate from milk precipitated by acid	trace	+
Alcoholic extract of whey powder	trace	+
Cod liver oil and the aqueous extract	+	++
Alcoholic and aqueous extract of Rous' chicken sarcoma	trace	++

We see from the table that the reactions are very specific for purine derivatives and polyphenols and they may therefore serve as a guide as to what groups the substances giving the reactions may belong. As the reactions are very sensitive, it seems to us that they might be used to ascertain the purity of phosphatides and other substances like caseinogen which are prepared from foodstuffs, and for which up to the present we possess no standard of purity. This test has already been found very useful in the investigation of the vitamine-fraction.

The table for purine derivatives shows that a substitution of one hydrogen atom in the purine ring lessens or destroys the power to give the uric acid reaction. In the case of the phenol reagent this is also brought about when two hydrogen atoms are substituted. The colour reactions with tyrosine derivatives and alloxantin were remarkably stable as compared with the others.

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XXXVI. THE PRODUCTION OF ACETALDEHYDE DURING THE ANAEROBIC FERMENTATION OF GLUCOSE BY BACILLUS COLI COMMUNIS (ESCHERICH).

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(*Received June 8th, 1913.*)

Acetaldehyde has long been known as a product of alcoholic fermentation by yeast [see Roeser, 1893]. It has likewise been found to be formed by the leaves of higher plants when their metabolism is restricted to anaerobic conditions [Kostytschew, 1913].

In view of this wide distribution, it was to be expected that acetaldehyde would be a product of bacterial fermentation. This is the case.

The quantity of acetaldehyde found by Roeser in various wine musts after a fermentation lasting from five to fifteen days, varied from 20–200 milligrams per litre of the fermented fluid. In the case of *B. coli communis* acting on glucose, one litre of fluid containing originally 15 grams of the sugar yielded in 15 days when half the sugar had been fermented 2·34 mg. of acetaldehyde, while, at the same time, 1·375 grams of alcohol had been produced. Since in the case of the true alcoholic fermentation quoted above, the quantity of alcohol formed was certainly twenty times that produced in the experiment with *B. coli communis*, it will be seen that the ratio of acetaldehyde to alcohol is of the same order in the two cases.

It is not at present settled, in the case of yeast fermentation, whether acetaldehyde is a primary product or whether it results from the oxidation of preformed alcohol, the following result is therefore significant in showing that the production of acetaldehyde by *B. coli communis* is related to the formation of alcohol, carbon dioxide, and acetic acid, rather than to the other products.

The experiment consisted in comparing the production of acetaldehyde from normal *B. coli communis* (Escherich) with that from a strain derived from the normal organism by selection with sodium chloracetate [see Penfold, 1911, and Harden and Penfold, 1912]. This derived strain had completely

lost the power of producing gas from carbohydrates. That it was closely related to the original organism was demonstrated by testing the agglutinating power of both the original and the derived organism towards a rabbit serum obtained after inoculating with the former. Both organisms agglutinated completely with the serum up to a dilution of one part of the serum in 25,600 of normal saline solution.

Reactions employed for the detection and determination of acetaldehyde.

(1) *Rimini's reaction* [1898].

A few drops of diethylamine are added to a similar quantity of a solution of sodium nitroprusside. A blue colour appears which rapidly fades (2–3 minutes). If this solution be now diluted with 1–2 c.c. of water a pale yellow or yellow-green solution is obtained but if diluted with 1–2 c.c. of a solution containing acetaldehyde a blue colour results characteristic of this aldehyde. Though specific for acetaldehyde the reagent is not so delicate as that of Schiff, and the coloration is moreover less permanent.

(2) *Schiff's reagent.* This reagent was used for the determination of acetaldehyde.

It is important to make sure that no alkali is present in the fluid to be tested by Schiff's reagent since even the alkalinity of tap water produces a definite coloration. The maximum intensity of colour, by simple addition of alkali, was obtained with 0·1 c.c. normal KHO, added to 50 c.c. distilled water containing 0·25 c.c. Schiff's reagent. A greater concentration of alkali destroys the colour, which is regained on the addition of acid. The solution before examination was therefore always tested with phenolphthalein paper.

Acetone is said to give a coloration with Schiff's reagent, but this effect which is not produced for many hours cannot possibly be confused with the reaction for acetaldehyde in which case the coloration reaches a maximum in about twenty minutes and has very considerably faded in less than an hour. It is important to emphasise this fact, since Mendel [1911] states that he found acetone amongst the products of the action of *B. coli communis* and certain other organisms on glucose.

Details of the determination of acetaldehyde.

(1) About 750 c.c. of the fluid containing the liquid and solid products of fermentation (acetaldehyde, alcohol, and calcium salts of volatile and non-volatile acids etc.) was treated with oxalic acid in excess of that necessary for

the complete precipitation of the calcium. When the precipitate of calcium oxalate had settled, as much as possible of the supernatant liquor was removed with the aid of a siphon, and distilled. The first 400–500 c.c. of the distillate was made alkaline with barium hydroxide and redistilled, using a fractionating column, until 100 c.c. had been collected. In both distillations the receivers were well cooled by means of ice.

50 c.c. of this solution were used for the determination of the acetaldehyde, the method of procedure being an adaptation of that of Ryffel [1909].

(2) A standard solution of formaldehyde was prepared as described by Ryffel. The strength of this solution was determined by comparing the colour produced from it on the addition of Schiff's reagent, with that produced on adding the same quantity of the reagent to a solution of acetaldehyde prepared by the distillation of a known weight of lactic acid.

0·02494 gram lactic acid was converted into acetaldehyde. The intensity of colour produced on adding 0·5 c.c. Schiff's reagent to 100 c.c. of this acetaldehyde solution was 1·9 times that of the standard formaldehyde colour as measured by a Duboscq tintometer, 100 c.c. of the standard formaldehyde solution was therefore equivalent to $\frac{0{\cdot}02494}{1{\cdot}9} = 0{\cdot}01312$ gram lactic acid.

Applying the ratio determined by Ryffel empirically for these conditions, viz.: 0·4 mg. formaldehyde = 3·435 mg. lactic acid = 1·765 mg. acetaldehyde, it follows that 100 c.c. of standard formaldehyde solution contains $\frac{0{\cdot}4 \times 0{\cdot}01312}{3{\cdot}435} = 1{\cdot}528$ mg. formaldehyde, and is equivalent to $\frac{1{\cdot}528 \times 1{\cdot}765}{0{\cdot}4} = 6{\cdot}471$ mg. acetaldehyde.

(3) 50 c.c. of the solution of formaldehyde described in paragraph (2) was diluted fivefold and compared with the distillate described paragraph (1), with the result:

The ratio of $\frac{\text{standard}}{5 \times \text{distillate}} = \frac{1}{1{\cdot}33}$.

From this it was calculated that the total amount of acetaldehyde in the original litre of fermented fluid was 2·34 mg.

Discussion of Results.

In order to demonstrate the relationship between the production of acet aldehyde and that of alcohol, carbon dioxide, hydrogen and acetic acid, the analyses of the products formed by the normal and the artificially selected strain are compared below, the results being calculated to 100 grams of the sugar.

	Products from glucose	
	Normal *B. coli communis* per cent.	Strain artificially selected by the chloracetate method per cent.
Hydrogen	0·42	Nil
Carbon dioxide	16·9	Nil
Alcohol	18·1	5·3
Acetic acid	18·5	10·8
Formic acid	9·7	11·1
Lactic acid	36·8	68·0
Succinic acid	0·7	0·8
Acetaldehyde	31·345 mg.	Nil
Glucose decomposed	7·465 gram	7·755
Acetaldehyde found	2·34 mg.	Nil

It will be seen that the artificially selected organism has (as in the case of the organism examined by Harden and Penfold), besides having lost the power of producing gas, also produced less alcohol and acetic acid. Coincidentally with these changes there has been an abolition or great reduction of the yield of acetaldehyde.

In the light of these results we may consider the question of the origin of the acetaldehyde.

If this substance were a secondary product formed by the oxidation of alcohol through the activity of oxidases concerned in the cell-growth as was suggested by Roeser for the case of yeast, then since there is a large excess of alcohol in both cases, the amount of acetaldehyde formed in the two experiments should be approximately the same, or at least, of the same order. Moreover in view of the fact that not only was air excluded from the fermentation flasks, but that in the experiment in which acetaldehyde was produced, 350 c.c. of hydrogen had been evolved, such an oxidation would seem unlikely to have occurred. The strongly reducing conditions of the experiment may be well seen from the experiment in which Harden attempted to employ asparagine as a source of nitrogen. This substance, he found, was completely reduced to ammonium succinate with corresponding diminution in the hydrogen evolved.

It may be suggested therefore that the production of acetaldehyde and part of the alcohol occur simultaneously. This would agree with the view held by Kostytschew [1912] that acetaldehyde is an intermediate product in alcoholic fermentation and would moreover suggest that part of the alcohol produced by the action of *B. coli communis* on glucose, passes through at least one of the same stages as that produced by the zymase of yeast.

From a consideration of the structure of glucose and mannitol, and the

fact that *B. coli communis* produces about twice as much alcohol from the latter as from the former, Harden [1901] suggested that the terminal group $-CH(OH).CH_2OH$ which occurs twice in mannitol though only once in glucose, is related to the production of alcohol.

Mannitol	$CH_2OH \cdot CHOH \cdot (CHOH)_2 \cdot CHOH \cdot CH_2OH$.
Glucose	$CH_2OH \cdot CHOH \cdot (CHOH)_2 \cdot CHOH \cdot CHO$.

If then the final group $-CHOH.CH_2OH$ conditions the production of alcohol, the corresponding group in glucose $-CHOH.CHO$ might stand in the same relation to acetaldehyde.

Clearly since an accumulation of this product would be harmful to the organism natural selection would have evolved the organism capable of transforming the aldehyde by reduction into ethyl alcohol, or oxidation to acetic acid.

Further results will be shortly forthcoming with regard to the production of acetaldehyde from other substances allied to glucose.

Summary.

(1) Acetaldehyde has been detected as a product of the action of *B. coli communis* on glucose, under anaerobic conditions.

(2) By artificial selection of *B. coli communis* by means of growth on sodium chloracetate, strains of the original organism have been obtained which produce either a greatly diminished amount of acetaldehyde or none at all.

(3) It has been found that the production of acetaldehyde is related to the formation of alcohol, carbon dioxide and hydrogen rather than to the other products. This has been ascertained by a comparison of the products formed by normal *B. coli communis* with those from an artificially selected strain produced by growth on agar containing sodium chloracetate.

(4) It is therefore suggested that acetaldehyde is a primary and not a secondary product of fermentation, and also that the process of alcohol formation by *B. coli communis* is in part analogous to the alcoholic fermentation set up by the zymase of yeast and to processes which occur in the leaves of higher plants.

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XXXVII. THE BIOCHEMICAL SYNTHESIS OF THE FATTY ACIDS.

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(*Received June 10th, 1913.*)

The methods by which fat is formed in the living organism remain at present completely unknown to us. Even the chemical reactions by which fatty acids are built up, comparatively simple though these must be, have hitherto found no satisfactory explanation. The one fact which appears to be completely established, by a large mass of experimental evidence, is that the carbohydrate of the food may be converted into fat inside the living organism, although neither the place where this change takes place nor the method by which it is accomplished is known with any degree of certainty.

The evidence as to the formation of fat from protein is less convincing, but it is at any rate possible that a conversion of protein into fat may also take place.

But little may be learnt from an attempt to correlate the composition of the fat stored up in the organism with the nature of the food supplied. There is abundant evidence that fatty acids taken in the food may be merely stored up unchanged in the body. The glycerides of palmitic, stearic and oleic acids are the constituents which most generally occur. Acids belonging to more highly unsaturated series than oleic acid have been demonstrated but these are more probably connected with further changes in the building up of fat into complex molecules, possibly of the nature of lecithin, than with the synthesis of the fatty acids themselves.

Two of the most prolific factories of fat are perhaps to be found (1) in plants, in such nuts as that of the cocoa-nut tree (*Cocos Nucifera*), where an abundant transformation of carbohydrate into fat must take place, and (2) in animals in the active mammary gland.

In both these instances, where a comparatively rapid conversion of

carbohydrate into fat is probably taking place the resultant fats are characterised by the presence of considerable quantities of the lower fatty acids. In cocoa-nut oil, the acids containing the even numbers of carbon atoms from six to eighteen, in butter from four to twenty, have been described. In these acids the carbon atoms are linked in straight chains and there is no evidence that any acid with a branched structure exists.

The question now arises whether the normal fatty acids present in butter are products of synthesis or of degradation. Knoop [1904] and Dakin [1908, 1909] have shown that the fatty acids are broken down by oxidation of the β-carbon atom; all the lower fatty acids present in butter may therefore be derived by oxidation from the arachidic or stearic acids present.

Some evidence on this point may be obtained from agricultural experiments; the problem has been directly investigated in an attempt to determine the reason of the variations which occur in the proportion of volatile fatty acids present in butter fat. Swaving (1906) carried out feeding experiments in the North of Holland to determine the cause of the low percentage of volatile soluble acids. Van der Zande and Siegfeld showed that a diet rich in carbohydrate, e.g. turnips, increased the proportion of the lower fatty acids and more recently Siegfeld [1907] and Amberger [1907] have shown that the increase is more especially in the insoluble volatile acids (i.e. caprylic, capric and lauric). Amberger, in a series of experiments carried out on the same set of cows showed that whereas food rich in protein such as malt germs diminishes the proportion of lower fatty acids, food rich in carbohydrate such as turnips increases this proportion. If the percentage of the lower fatty acids increases with the amount of the carbohydrate in the food, it would appear more probable that they exist as intermediate synthetic products on their way to the higher fatty acids, than as degradation products. Such evidence as exists is therefore in favour of a synthesis in which all fatty acids containing even numbers of carbon atoms from four to twenty linked together in straight chains are formed from carbohydrate in some way through the agency of the mammary gland.

Previous Hypotheses as to the Nature of the Reactions by which Fatty Acids are formed from Carbohydrate in the Animal Organism.

Emil Fischer suggested that stearic and oleic acids are formed by the condensation of three hexose molecules or of six triose (glycerose) molecules in such a way that a straight chain containing 18 carbon atoms

is formed. From this, by further processes of oxidation and reduction, stearic and oleic acids are formed. Palmitic acid with its chain of 16 carbon atoms would be compounded from two pentose and one hexose molecules. Glucose, gluconic and glucuronic acids are suggested as the precursors of the pentose molecules. In favour of this hypothesis it is difficult to find any evidence either of a chemical or biological nature. Against it the following considerations may be urged:

(*a*) No laboratory method is known by which two hexose molecules may be made to condense in such a way that a straight chain of twelve carbon atoms is produced.

(*b*) Pentoses are known to exist in the organism in combination in the nucleoproteins but there is no indication that the pentoses are in any way connected with the formation of fat or with normal carbohydrate metabolism.

(*c*) If it be granted that the fatty acids of butter are products formed synthetically from carbohydrate, the hypothesis presents insuperable difficulties. No combination of hexose and pentose molecules will produce myristic acid ($C_{14}H_{28}O_2$) by direct addition, yet this acid occurs commonly in fats, e.g. lard, butter, cod-liver oil and many vegetable fats. The existence of an intermediate tetrose sugar would have to be assumed as a normal constituent.

3 hexose molecules	give	stearic acid.
2 pentose and one hexose molecules	,,	palmitic acid.
2 pentose and one tetrose ,,	,,	myristic acid.
2 hexose molecules	,,	lauric acid.
2 pentose ,,	,,	capric acid.
2 tetrose ,,	,,	caprylic acid.

There is no evidence of the formation of a tetrose molecule in the organism and it is exceedingly unlikely that a regular series of fatty acids should be formed in this way.

(*d*) In the case of stearic acid the reduction of seventeen hydroxyl groups would be assumed.

This hypothesis has therefore no evidence in its favour and involves reactions which are not analogous with any of those known to us. It does not therefore furnish us with a satisfactory explanation of the problem under consideration.

The second hypothesis, which is perhaps the more generally accepted, is that the fatty acids are built up by repeated condensations of a compound containing two carbon atoms. This was first suggested by Nencki and afterwards developed by Magnus-Levy [1902], Leathes and others who regarded acetaldehyde as the substance from which by a series of aldol condensations

the straight chains containing even numbers of carbon atoms were formed. The reactions involved would be represented by the following equations:

(1) $CH_3.CHO + CH_3.CHO = CH_3.CHOH.CH_2.CHO$

(2) $CH_3.CHOH.CH_2.CHO = CH_3.CH_2.CH_2.COOH + H_2O$

$+ \begin{matrix} H \\ H \end{matrix} \!\!>\!\!\!> O.$

If the aldol on the other hand were reduced to butyl aldehyde, it would be available again to take part in a similar condensation:

$$CH_3.CHOH.CH_2.CHO + H_2 = CH_3.CH_2.CH_2.CHO + H_2O$$
$$CH_3.CH_2.CH_2.CHO + CH_3.CHO = CH_3.CH_2.CH_2.CHOH.CH_2.CHO.$$

a normal six-carbon-atom chain being thus produced.

In favour of this hypothesis, it may be urged:

(*a*) It does account for the production only of those fatty acids containing even numbers of carbon atoms, since only multiples of two will exist.

(*b*) Hoppe-Seyler showed that by the action of caustic alkali on lactic acid at from 200°–300°, acetic, butyric and caproic acids were formed. Pasteur had previously shown that butyric and caproic acids were formed by the bacterial fermentation of sugar. Acetic aldehyde may be obtained from lactic acid and may therefore be a degradation product of sugar.

On the other hand it is open to the following criticisms:

(*a*) Lieben [1883, 1901] and his pupils have shown that when the higher aldehydes condense with acetaldehyde under the influence of dilute alkalies, the resulting aldehydes possess a branched and not an open-chain structure:

$$CH_3.CH_2.CH_2.CHO + CHO.CH_3 \longrightarrow CH_3.CH_2.CH.CHO$$
$$\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\quad |$$
$$\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\quad CHOH$$
$$\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\quad |$$
$$\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\quad CH_3.$$

It has since been shown that both aldol and crotonaldehyde will undergo auto-condensation with the formation of a normal eight-carbon-atom chain [Raper, 1907; Smedley, 1911]; but the difficulty of adding on acetic aldehyde to a higher aldehyde so as to build up chains increasing by the addition of two carbon atoms has not been surmounted. One must therefore assume that the condensation of aldehydes in the body does not take place in the same manner as it does when brought about by the action of condensing agents in the laboratory.

(*b*) No free aldehydes other than the sugars have been detected in the body. If present in quantity they would probably be injurious to the life of the cell. Parnas [1910] has shown that an enzyme is present in the liver by which free aldehydes are at once removed.

(*c*) There is no biological evidence that acetaldehyde is formed as an intermediate substance in the body metabolism.

The aldol condensation does not therefore furnish us with a satisfactory analogy for the method by which the fatty acids are built up.

A survey of the general methods of producing fatty acids in the laboratory shows that the most satisfactory method by which fatty acids may be built up by increments of two carbon atoms is by means of Reformatski's reaction in which aldehydes are condensed with bromoacetic ester in the presence of zinc;

$$R.CHO + BrCH_2.COOEt + Zn + H_2O = R.CHOH.CH_2.COOEt + Zn\begin{matrix}OH\\Br\end{matrix}.$$

As however neither zinc nor bromoacetic ester occurs in the body, this does not furnish us with any helpful analogy for biochemical synthesis.

The Degradation-products of Carbohydrates: their suitability as Units in the biochemical synthesis of Fatty Acids.

But little is known as to the manner in which carbohydrate breaks down within the body. It has been repeatedly established that when a solution of glucose in Ringer's fluid is perfused through the isolated heart sugar disappears [Locke and Rosenheim, 1907; MacLean and Smedley, 1913]. This is the only instance in which it has been established beyond the region of controversy that sugar disappears when subjected to the action of an isolated organ. But even here the decomposition products of the sugar molecules are unknown. The controversy as to whether glycogen is a storage product or a stage in the normal metabolism of sugar throws little light on the problem under consideration. The discussion as to whether glucose is the source of the lactic acid in the animal organism has more bearing on the subject of fat formation. Embden has shown that the transfusion of blood rich in sugar through a glycogen-free liver resulted in the abundant formation of lactic acid: blood poor in sugar similarly transfused gave rise to lactic acid in inconsiderable amount. The formation of lactic acid from carbohydrate is also indicated by the experiments of Mandel and Lusk on phlorizin diabetes. It seems probable that both carbohydrate and protein may give rise to lactic acid in the body. The occurrence of lactic acid as a possible cleavage product of carbohydrate suggests that the breaking down of sugar takes place in such a way as to give rise to compounds containing three carbon atoms. Lactic acid itself is not a very reactive substance nor does

it appear a hopeful starting material for the synthesis of fatty acids. It is however closely related to pyruvic acid.

$CH_3.CHOH.COOH$	$CH_3.CO.COOH$
Lactic Acid	Pyruvic Acid

There is also evidence that pyruvic acid itself is probably of considerable importance in animal metabolism. It has been demonstrated that a close connection exists in the organism between the α-amino- and the α-keto-acids. Embden and Schmitz [1910, 1912] have shown that if a solution of ammonium pyruvate be perfused through a liver, alanine is formed. Fellner [1912] further showed that if a liver rich in glycogen be perfused with blood containing ammonia, alanine is formed, and from a consideration of Embden's experiments pyruvic acid is indicated as the intermediate substance. Neubauer and Knoop and Kertess [1911] have also suggested the formation of alanine from pyruvic acid in the body.

Knoop [1910] and Knoop and Kertess [1911] have shown that if γ-phenyl-α-amino-butyric acid be fed to a dog, a considerable proportion of the acid appears in the urine as the acetyl derivative; the same phenomenon was observed by Neubauer and Warburg [1910] in their perfusion experiments. There is some reason to believe that the acetylating agent may be pyruvic acid, since de Jong [1900, 1904] showed that ammonium carbonate and pyruvic acid react with formation of acetyl-alanine. It seems therefore probable that pyruvic acid may be an intermediate substance formed in the body from carbohydrate.

Pyruvic acid is a reactive substance, readily losing carbon dioxide under the influence of oxidising agents and forming acetic acid. A study therefore of its chemical properties and of its powers of condensation seemed of especial interest.

The condensation of Pyruvic Acid with Fatty Aldehydes and the oxidation of the products formed.

It had already been shown that if anhydrous hydrochloric acid be passed into a mixture of benzaldehyde and pyruvic acid, cinnamoyl-formic acid results [Erlenmeyer, 1901];

$$C_6H_5.CHO + CH_3.CO.COOH = C_6H_5.CH:CH.CO.COOH + H_2O.$$

Later both benzaldehyde and cinnamyl aldehyde were condensed with pyruvic acid by adding a small amount of 10% caustic soda to the mixture [Erlenmeyer, 1903].

In order to make use as far as possible only of reagents which may be considered to bring about reactions somewhat similar to those brought about

by enzymes within the body, the condensation of the fatty aldehydes with pyruvic acid was attempted in very dilute alkaline solution. The intermediate unsaturated α-keto acid which was expected to result was not isolated, but the product was at once oxidised by silver oxide in alkaline solution or by hydrogen peroxide in neutral solution.

The behaviour of crotonaldehyde was first investigated.

Experimental.

Condensation of crotonaldehyde with pyruvic acid and oxidation of the product formed.

5 grams pyruvic acid, 5 grams crotonaldehyde, 75 cc. n. NaOH and 1 litre of water were added together and left for three days at the room temperature, the solution being approximately 1/50 normal. The liquid became deep yellow but no insoluble oil separated as in the condensation of crotonaldehyde alone. The solution was neutralised by the addition of 12·5 cc. n. H_2SO_4 and steam distilled to remove any free aldehyde.

Oxidation of Reaction-product.

Silver oxide was precipitated from 30 grams silver nitrate and added to the solution of the condensation product of crotonaldehyde with pyruvic acid after it had been steam distilled. 200 cc. of a 1/3 normal solution of baryta were gradually added and the whole allowed to stand over night. Next morning the silver oxide had been largely converted to silver. The precipitate was filtered off, washed and concentrated under reduced pressure until 50 cc. remained. In order to convert any hydroxy-acid that might conceivably be present to the corresponding unsaturated acid, 10 grams of baryta were added and the mixture boiled for 30 minutes.

Excess of sulphuric acid was then added and the whole steam-distilled. 1500 cc. of the steam distillate required 28·5 cc. normal potash for neutralisation. The neutral distillate was evaporated almost to dryness and to the potassium salt so obtained, 10 % H_2SO_4 was added. Crystals separated which melted at 132° after once recrystallising from dilute alcohol. The melting point was unchanged on mixing with a specimen of sorbic acid prepared by the condensation of crotonaldehyde and bromoacetic ester, and hydrolysis of the ester formed. The crystals were therefore satisfactorily identified as sorbic acid.

In subsequent experiments the oxidation of the neutral condensation product was carried out by means of hydrogen peroxide. An amount of hydrogen peroxide exactly equivalent to the pyruvic acid originally taken

was used and the neutral mixture of condensation product and peroxide left to stand over night at the ordinary temperature; the product was concentrated under reduced pressure and steam distilled as before, and the final product consisted of a mixture of acetic and sorbic acids, the yield being somewhat improved by this means. From 5 grams of crotonaldehyde 0·5 g. sorbic acid was thus obtained.

The reaction must therefore have proceeded as follows:

$$CH_3.CH:CH.CHO + CH_3.CO.COOH = CH_3.CH:CH.CH:CH.CO.COOH$$
$$CH_3.CH:CH.CH:CH.CO.COOH + H_2O_2 = CH_3.CH:CH.CH:CH.COOH + H_2O + CO_2$$
Sorbic Acid.

The condensation of Butyl Aldehyde with Pyruvic Acid.

10 grams of butyl aldehyde, 10 grams of pyruvic acid and 150 cc. normal potash were shaken up with 2 litres of water and at the end of 12 days the mixture was neutralised and concentrated under diminished pressure. To the concentrated residue silver oxide from 43 grams of silver nitrate and 200 cc. n/3 baryta were added. After standing over night, the silver precipitate was filtered off and the filtrate concentrated to 250 cc., 50 grams of baryta added and boiled for 30 minutes. The whole was then acidified with dilute sulphuric acid and distilled in steam. 1500 cc. of distillate were neutralised by 59·2 cc. normal potash and evaporated to dryness. The residue was acidified and extracted with ether. After evaporating off the ether, the residue was distilled under a pressure of 20 mm. About 3 grams boiling from 130°–140° were obtained. The liquid rapidly decolourised bromine water and gave on analysis the numbers required for the compound $C_6H_{10}O_2$.

0·1409 g.; 0·3262 g. CO_2; 0·1134 g. H_2O.
C 63·14 % H 8·94 %.
Calc. for $C_6H_{10}O_2$ C 63·15 % H 8·77 %.

In subsequent experiments, the product similarly prepared appeared to consist of a mixture of octylenic acid (probably obtained by the self-condensation of the butyl aldehyde) and of hexylenic acid obtained from butyl aldehyde and pyruvic acid. The difficulty of separating these in a small quantity of a liquid mixture is considerable.

In another experiment where hydrogen peroxide was used as the oxidising agent as described under crotonaldehyde, the product obtained distilled under reduced pressure (15 to 20 mm.) from 120°–128° and gave on analysis the following numbers.

0·1220 g.; 0·2858 g. CO_2: 0·1074 g. H_2O.
C 63·85 %: H 9·75 %.

In investigating the condensation of iso-valeraldehyde and oenanthol with pyruvic acid, chiefly the products of condensation of the aldehydes with themselves were isolated. From the condensation product of iso-valeraldehyde and pyruvic acid, a small amount of the barium salt of an acid was obtained, the percentage of barium in which agreed with that required for the barium salt of the corresponding unsaturated keto-acid.

Condensation of these higher fatty aldehydes with pyruvic acid under varying conditions is now being investigated. The condensation of croton and butyl aldehydes with pyruvic acid and the oxidation of the product formed with hydrogen peroxide in neutral solution furnishes a method by which an unsaturated fatty acid may be built up containing two more carbon atoms than the aldehyde from which it is derived. These condensations have also been investigated under similar conditions in the aromatic series [Smedley and Lubrzynska, 1913].

Conclusions.

The hypothesis now brought forward [Smedley, 1912] suggests that pyruvic acid, formed in the body as a decomposition product of carbohydrate, is the starting-point for the synthesis of the fatty acids. The stages which are assumed to occur are represented by the following equations:

(1) $CH_3.CO.COOH = CH_3.CHO + CO_2$.
Pyruvic Acid → Acetaldehyde.

(2) $CH_3.CHO + CH_3.CO.COOH = CH_3.CHOH.CH_2.CO.COOH$
$CH_3.CH:CH.CO.COOH + H_2O$.
Pentylenic α-keto-acid.

(3a) $CH_3.CH:CH.CO.COOH + O = CH_3.CH:CH.COOH + CO_2$.

(4a) $CH_3.CH:CH.COOH + 2H = CH_3.CH_2.CH_2.COOH$.
Butyric Acid.

(3b) $CH_3.CH:CH.CO.COOH = CH_3.CH:CH.CHO + CO_2$.
Pentylenic α-keto acid.

(4b) $CH_3.CH:CH.CHO + CH_3.CO.COOH = CH_3.CH:CH.CHOH.CH_2.CO.COOH$.

and by reactions similar to 3*a* and 4*a*

$CH_3.CH_2.CH_2.CH_2.CH_2.COOH$.
Caproic acid.

The evidence supporting this hypothesis may be briefly summarised as follows.

1. Pyruvic acid is probably a degradation product of carbohydrate in the body.

The perfusion experiments of Embden, Knoop and Neubauer show that pyruvic acid is converted into alanine through the agency of the liver cells

and that a close connection exists between the α-amino- and α-keto-acids. Pyruvic acid may probably be an intermediate stage in the transformation from glycogen to alanine (Fellner).

There is some reason to believe that in the acetylation of certain amino-acids which has been observed both in perfusion and in feeding experiments, pyruvic acid is the acetylating agent.

The close connection between alanine and pyruvic acid suggests that the alanine group of the protein molecule may furnish an additional source of the pyruvic acid available for the synthesis of fatty acids.

2. The decomposition of pyruvic acid into acetaldehyde and carbonic acid, which constitutes the first stage of this process, has been shown by Neuberg to be readily brought about by an enzyme present in yeast, termed "carboxylase."

3. The present hypothesis postulates that free acetaldehyde is not liberated but that the decomposition of the keto-acid is in some way regulated by the pyruvic acid with which the "nascent" aldehyde combines.

The condensation of fatty aldehydes with pyruvic acid has now been shown to take place in the laboratory under the influence of dilute alkalis at ordinary temperature.

4. Oxidation of the α-keto acid according to the equation

$$R.CO.COOH + O = R.COOH + CO_2$$

may be brought about in the laboratory by hydrogen peroxide at the ordinary temperature in neutral solution (p. 370).

5. The reduction of the unsaturated acid is the final stage; there is abundant evidence that reduction can take place in the body although very little is known as to the mechanism by which it is accomplished.

6. The α-keto-acid, synthesised as above, may be split into CO_2 and aldehyde, and a further condensation with pyruvic acid may then be effected. An acid with two more carbon atoms than the original aldehyde would thus be synthesised.

As yet no α-keto-acids have been detected within the body: it may be that they occur only within the cell and that reduction or oxidation always accompanies their liberation. The above hypothesis accounts for the formation of a series of straight chain acids beginning with four carbon atoms and increasing by increments of two carbon atoms: it involves only reactions which are analogous with those which are known to occur in the laboratory and there is reasonable evidence for believing that the starting material, pyruvic acid, can be formed from carbohydrate in the body.

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XXXVIII. THE CONDENSATION OF AROMATIC ALDEHYDES WITH PYRUVIC ACID.

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In a preliminary communication published by one of us [Smedley, 1912], and in the foregoing paper [Smedley and Lubrzynska, 1913], the condensation of certain fatty aldehydes with pyruvic acid in very dilute alkaline solution has been described.

In order to obtain a better knowledge of the reaction and hence possibly to overcome some difficulties with which we found ourselves confronted in our experiments in the aliphatic series we have investigated the behaviour of certain aromatic aldehydes under similar conditions. A number of the $\beta\gamma$-unsaturated α-keto-acids have been prepared and converted by oxidation with hydrogen peroxide into the corresponding unsaturated acids containing one carbon atom less than the original keto-acid.

Claisen and Claparède [1882] studied the action of anhydrous hydrochloric acid on a mixture of benzaldehyde and pyruvic acid and obtained from the mixture cinnamoyl formic acid. This reaction was further studied by Erlenmeyer, junr. [1899, 1901], who showed that in addition to the above product γ-phenyl-β-benzylidene-α-ketobutyrolactone is formed:

$$C_6H_5.CHO + CH_3.CO.COOH = C_6H_5.CH:CH.CO.COOH + H_2O.$$
$$2C_6H_5.CHO + CH_3.CO.COOH = C_6H_5.CH:\underline{C.CO.CO.O.CH}.C_6H_5 + 2H_2O.$$

The condensations of benzaldehyde, cinnamic aldehyde, piperonal and anisic aldehyde with pyruvic acid have been studied in dilute alkaline solution. The reactions proceed in all cases readily at the laboratory temperature. The amount of potash used was generally such that the strength of the solution was from 1/40 to 1/10 normal, and the mixture was allowed to stand at the temperature of the laboratory for periods varying from 2 to 7 days. As the reaction proceeds the aldehyde disappears and the

liquid gradually becomes yellow. When the reaction is completed, the keto-acid is thrown down on acidifying the liquid, and after purification, is oxidised to the corresponding $\alpha\beta$-unsaturated acid.

Yellow crystalline products are formed by the condensation of anisic aldehyde and piperonal respectively with pyruvic acid, the formulae of which are shown on analysis to agree with those required for the β-unsaturated α-keto-acids:

$$R.CHO + CH_2.CO.COOH = R.CH:CH.CO.COOH + H_2O.$$
$$R.CH:CH.CO.COOH + H_2O_2 = R.CH:CH.COOH + CO_2 + H_2O.$$

After we had completed the above series of experiments, we found that we had overlooked two papers by Erlenmeyer, junr. [1903, 1904], in which he had described the condensation of benzaldehyde and cinnamic aldehyde respectively with pyruvic acid in the presence of strong caustic soda (10 %). The products which we obtained on condensing cinnamic aldehyde with pyruvic acid were similar to those described by Erlenmeyer. He isolated two forms, an orange-red, melting at 75° and gradually converted *in vacuo* into a yellow form melting at 107°. Our products melted at 73° and 104° (uncorrected) respectively.

From anisic aldehyde and pyruvic acid yellow crystals melting at 130° were obtained. Piperonal under similar conditions gives beautiful yellow needles which at a temperature of about 70° undergo a remarkable transition into a deep orange-red form and finally melt at 163°. On keeping the red form so obtained in an evacuated desiccator or in a well-stoppered bottle it was still unchanged after a month had elapsed. If however it was exposed to the air it was gradually reconverted into the yellow variety.

On oxidation with the calculated quantity of hydrogen peroxide in neutral solution at the ordinary temperature the keto-acids split off carbon dioxide and form unsaturated acids:

$$R.CH:CH.CO.COOH + H_2O_2 = R.CH:CH.COOH + CO_2 + H_2O.$$

Silver oxide which was successfully used to oxidise the corresponding keto-acids in the fatty series [Smedley, 1912], was found to be without action in the aromatic series.

Experimental.

Condensation of piperonal with pyruvic acid. 4 g. of pyruvic acid and 6 g. of piperonal were shaken up with 1 litre of water and 140 cc. N. KOH added; the mixture was allowed to stand at the ordinary temperature for a week, during which it became yellow. The small amount of unchanged

piperonal was filtered off and the solution neutralised with 36 cc. N. H_2SO_4. The neutral solution was extracted with ether several times to free it from traces of unchanged aldehyde. Air was drawn through to remove the last traces of ether and the solution acidified. A yellow solid was precipitated and allowed to settle: it was then filtered off and well washed with cold water. After several recrystallisations from dilute alcohol it was obtained in beautiful yellow needles which turned to deep orange-red at about 70° and melted at 163°.

0·1062 g.; 0·2336 g. CO_2; 0·0368 g. H_2O.
C 59·98 %; H 3·85 %.
Calc. for $C_{11}H_8O_5$, C 60·00 %; H 3·63 %.

Condensation of anisic aldehyde with pyruvic acid. 5 g. anisic aldehyde and 3·5 g. pyruvic acid were shaken up with 1 litre of water and 70 cc. N. KOH added, the mixture being allowed to stand for 4 days during which a yellow colour developed. It was then neutralised with 25·05 cc. N. H_2SO_4 and the mixture treated exactly as in the case of the condensation of piperonal with pyruvic acid.

The yellow needles finally obtained melted at 130° and no change in colour was observed either on standing or on heating.

0·1100 g.; 0·2585 g. CO_2; 0·0500 g. H_2O.
C 64·09 %; H 5·00 %.
Calc. for $C_{11}H_{10}O_4$, C 64·07 %; H 4·85 %.

The specimen, part of which gave the above results on analysis, was allowed to remain in a closed test-tube for two weeks and then analysed:

0·0972 g.; 0·2330 g. CO_2; 0·0407 g. H_2O.
C 65·32 %; H 4·63 %.

A week later it was again analysed:

0·1208 g.; 0·2927 g. CO_2; 0·0562 g. H_2O.
C 65·98 %; H 5·16 %.

The acid was then recrystallised and again analysed:

0·1244 g.; 0·2972 g. CO_2; 0·0564 g. H_2O.
C 65·11 %; H 5·06 %.

The preparation was repeated and the freshly prepared product analysed:

0·1169 g.; 0·2743 g. CO_2; 0·0518 g. H_2O.
C 64·10 %; H 4·91 %.

The acid therefore on standing undergoes a slight decomposition, the nature of which was not ascertained.

Condensation of cinnamic aldehyde with pyruvic acid. 5 g. cinnamic aldehyde and 4 g. pyruvic acid were shaken up with 1 litre of water and 80 cc. of normal potash. The mixture was allowed to stand for 4 days at the

ordinary temperature, neutralised and treated as in the experiments already described with piperonal and anisic aldehyde.

The condensation product was obtained in two forms; red crystals melting at 73°, which pass gradually into a yellow substance melting at 104°. In one experiment red crystals melting at 73° were exposed in an evacuated desiccator for 48 hours: they had then become yellow and melted at 95°. After another 24 hours the melting point was 101° and after recrystallisation from dilute alcohol it rose to 103°. Analysis showed that the composition of the two forms after drying in a vacuum desiccator was identical.

0·1274 g.; 0·3344 g. CO_2; 0·0600 g. H_2O.
C 71·58 %; H 5·17 %.
Calc. for $C_{12}H_{10}O_3$, C 71·28 %; H 4·95 %.

Oxidation of dioxymethylene-benzylidene-pyruvic acid:

$CH_2O_2C_6H_3CH : CH . CO . COOH$.

The keto-acid was dissolved in alcohol, neutralised with potash and the theoretical quantity of hydrogen peroxide then added. It is important that no excess of the peroxide be present or the yield will be found to be diminished. The oxidation product was heated for an hour on a water-bath to complete the reaction and then acidified. A very pale yellow acid was precipitated, which after one recrystallisation was almost white and melted at 242°.

0·1074 g.; 0·2462 g. CO_2; 0·0423 g. H_2O.
C 62·47 %; H 4·37 %.
0·1148 g.; 0·2690 g. CO_2; 0·0458 g. H_2O.
C 62·51 %; H 4·32 %.
Calc. for $C_{10}H_8O_4$, C 62·50 %; H 4·16 %.

Oxidation of cinnamylidene-pyruvic acid:

$C_6H_5 . CH : CH . CH : CH . CO . COOH$.

The oxidation was carried out in a manner similar to that just described for the piperonal product. The acid obtained after recrystallisation from benzene and from alcohol melted at 165° and was therefore identical with the cinnamylidene-acetic acid prepared by Perkin from malonic acid and cinnamic aldehyde, melting at 165°.

0·0844 g.; 0·2335 g. CO_2; 0·0442 g. H_2O.
C 75·47 %; H 5·80 %.

After again recrystallising:

0·1093 g.; 0·3052 g. CO_2; 0·0578 g. H_2O.
C 76·12 %; H 5·85 %.
Calc. for $C_{11}H_{10}O_2$, C 75·86 %; H 5·74 %.

Oxidation of methoxy-benzylidene-pyruvic acid:

$CH_3O . C_6H_4 . CH : CH . CO . COOH$.

This acid was dissolved in alcohol, carefully neutralised with potash and hydrogen peroxide added. In this case it was found that the best yield was obtained on adding 1·5 times the theoretical quantity of the peroxide. After several recrystallisations, the acid melted at 172°. Analysis gave the following result:

0·1020 g.; 0·2517 g. CO_2; 0·0530 g. H_2O.
C 67·23 %; H 5·70 %.
Calc. for $C_{10}H_{10}O_3$, 67·41 %; H 5·62 %.

Condensation of benzaldehyde with pyruvic acid. The condensation was carried out as in the cases already described, but the intermediate keto-acid was not isolated. The product was at once oxidised in neutral solution with hydrogen peroxide.

It was found in this case of the greatest importance to have the solution exactly neutral and to avoid any excess of the peroxide.

On acidification, cinnamic acid was precipitated. It was identified by taking the melting-point of a specimen mixed with pure Kahlbaum's cinnamic acid.

The yields in the above experiments varied from 50–70 % of the theoretical.

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XXXIX. THE PRECIPITATION OF EGG-ALBUMIN BY AMMONIUM SULPHATE. A CONTRIBUTION TO THE THEORY OF THE "SALTING-OUT" OF PROTEINS.

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Introduction.

The first systematic and quantitative researches upon the precipitation of proteins by salts in large amounts were made by Hofmeister and his pupils.

In the experiments of Kauder [1886] and Lewith [1888] with serum proteins, and Hofmeister [1888] with egg-protein, comparison was made of the precipitating power of a large series of electrolytes, as a result of which the latter were arranged in what is known as the Hofmeister series. Most of the experiments were made with sodium salts, among which sulphate, phosphate, acetate, citrate, tartrate, chromate, chloride, nitrate and chlorate formed a series arranged in descending power of precipitation; of the kations examined lithium was the most effective, and sodium, potassium, ammonium and magnesium came afterwards in order of decreasing efficiency.

Hofmeister [1889] came to the conclusion that the precipitation was caused by the electrolytes depriving the protein of the amount of water necessary to keep it in solution, and was confirmed in this view by the results of some experiments showing the influence of various salts in modifying the imbibition of water by gelatin [1891]; work in this direction was later extended by Pauli [1898] and Pauli and Rona [1902].

Spiro [1904] demonstrated that the precipitation from their solutions of caseinogen and gelatin by sodium sulphate was analogous to the "salting out" of alcohol recently studied in detail by de Bruyn [1900]. In neither case is the phenomenon one of simple precipitation, since, owing to the appropriation of water by the salt, separation into two phases occurs. Each phase contains all the constituents of the system and any alteration in one of

the three constituents leads to readjustment of the composition and relative volumes of the two phases. Spiro also pointed out that, since in the case of alcohol the effect of electrolytes is not attributable to the constituent ions, any influence of the latter in the salting out of proteins must be regarded as a subsidiary phenomenon.

Spiro's conception explains to some extent the divergent results obtained in the precipitation of proteins by the addition of neutral salts, when the whole conditions are not maintained constant.

The series of observations which we are about to record concern the "salting out" by ammonium sulphate of pure recrystallised egg-albumin. Our observations show that in this case also we have to deal with the separation of the original system (itself not homogeneous) into two distinct phases, and that the influence upon the volume of these phases of concentration of protein, salt and water in the system is, as Spiro found for caseinogen and gelatin, analogous to what occurs in alcohol, salt and water mixtures. In addition, however, we find that the charge carried by the protein particles is an important factor in the final equilibrium.

The results of the experiments will first be set forth and the proposed explanation discussed later.

Precipitation of pure egg-albumin by ammonium sulphate.

Material. The material employed was egg-albumin crystallised from egg-white in presence of ammonium sulphate according to the method of Hopkins and Pinkus [1898]. The albumin was recrystallised once or twice, separated from the mother liquor by pressing between filter paper, and finally dissolved in distilled water. A concentrated stock solution was thus obtained, the composition of which, as regards (1) protein, (2) ammonium sulphate, (3) water was accurately ascertained by analysis, and which, when diluted to a suitable degree, served for most of the following experiments.

Since the salt employed for "salting out" in these experiments was also ammonium sulphate, the small concentration of the latter always present in the original albumin solution presented no complication; an allowance was made for this amount in the calculations. In those cases where an electrolyte-free solution was required, the albumin solution was previously dialysed.

Egg-albumin prepared in this way we believe to be as homogeneous a protein as it is possible to obtain. Hopkins [1899–1900] came to the conclusion that egg-albumin, crystallised from faintly acid ammonium sulphate solutions by the above method, was a pure substance. The

rotatory power remained absolutely constant after repeated recrystallisations (p. 312) and the proportion of carbon, hydrogen, nitrogen and sulphur, as well as the ash, remained constant. His experiments were made with four different samples after three or four recrystallisations.

1. *Influence of concentration of salt upon the amount of protein precipitated.*

Mellanby [1907] made a quantitative study of the influence of concentration of ammonium sulphate on the precipitation of the proteins from horse serum, but, as far as we are aware, no experiments have as yet been made with a pure protein. In the present instance two sets of experiments were made, in both of which the concentration of protein was about 1 %. In the first the concentration of protein in the whole system was left constant = 1·11 % by weight, and the ratio salt to water was varied (Table I and Fig. 1). In the second set the ratio protein to water was kept constant and the precipitation studied by varying the amount of salt present; the concentration of protein in the whole system varied from 1·0 % to 0·93 % (by weight). (Table II and Fig. 2.)

TABLE I.

Precipitation of pure egg-albumin with ammonium sulphate; influence of concentration of salt.

Temperature, 20°.
Protein constant = 1·11 % by weight of total system.
Ratio salt/water varying.

Albumin g.	Water g.	Salt g.	G. albumin in 100 g. total system	G. salt in 100 g. total system	G. albumin in 100 g. filtrate
1·00	69·00	20·00	1·11	22·22	1·089
1·00	68·00	21·00	,,	23·33	0·711
1·00	67·00	22·00	,,	24·44	0·302
1·00	66·00	23·00	,,	25·55	0·104
1·00	65·00	24·00	,,	26·66	0·0315
0·50	32·35	12·15	,,	27·00	trace
0·50	32·00	12·50	,,	27·77	trace

The method of experiment was as follows. Mixtures were prepared by weighing into stoppered bottles the required amount of water and protein, and the necessary amount of ammonium sulphate was then added in large crystals and gently shaken. This prevented over-saturation with ammonium sulphate in the neighbourhood of the crystals, the large size of which

prevented a too rapid solution. The bottles were placed in a thermostat at 20°, for from 1 to 2 hours, the contents filtered and the protein estimated in the filtrate by weighing the coagulum formed on heating.

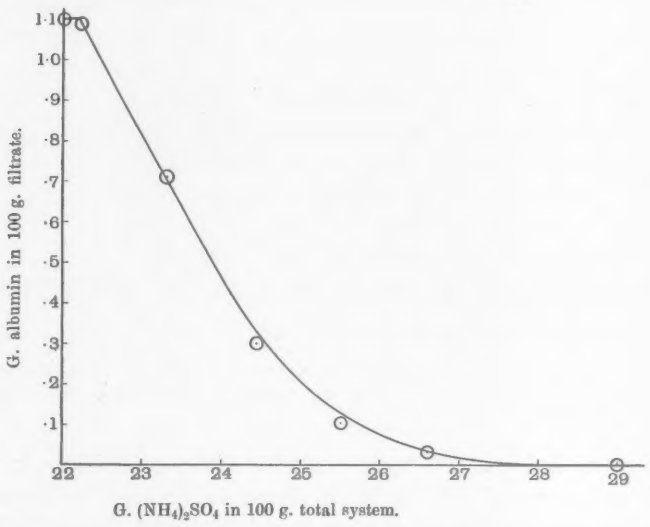

Fig. 1. Influence of concentration of the salt upon precipitation of egg-albumin by $(NH_4)_2SO_4$ at 20°, see Table I; protein constant = 1·11 % (by weight) of total system, ratio salt to water varying.

TABLE II.

Precipitation of pure egg-albumin with ammonium sulphate; influence of concentration of salt.

Temperature, 20°.
Ratio protein/water constant = 1·3/100.

Albumin g.	Water g.	Salt g.	G. salt to 1·3 g. protein and 100 g. H_2O	G. albumin in 100 g. filtrate
1·30	100	29	29	0·998
1·3	100	29·7	29·7	no precipitation.
1·30	100	30	30	0·938
1·95	150	46·5	31	0·733
1·3	100	32	32	0·487
2·6	200	66	33	0·273
2·6	200	67	33·5	0·232
2·6	200	70·01	35·0	0·105
2·6	200	76	38·0	0·022

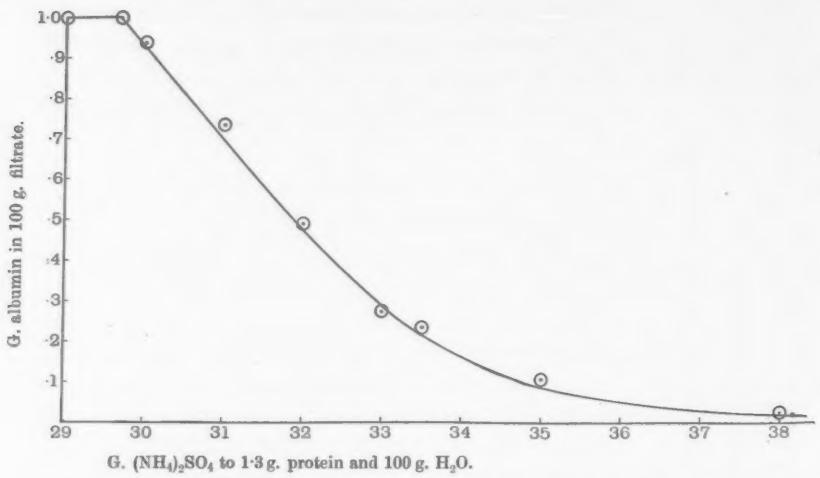

Fig. 2. Influence of concentration of the salt upon the precipitation of egg-albumin by $(NH_4)_2SO_4$ at 20°, see Table II; ratio protein to water constant = 1·3 to 100, amount of salt present varying.

The curves in Figs. 1 and 2 are both of the same general form, in both cases the percentages of albumin in the filtrate are plotted as ordinates and the abscissae are respectively percentage by weight of ammonium sulphate in the whole system and grams of ammonium sulphate present, which explains the fact that the curve is steeper in the former instance. The point of commencing precipitation which is very sharply marked is at about the same concentration of salt in both cases, viz. 22·2 and 22·7 % ammonium sulphate respectively; the curve then descends steeply and approaches the base line asymptotically.

2. *Influence of concentration of protein.*

Kauder [1886] showed that serum albumin was more readily precipitated by ammonium sulphate if in more concentrated solution and determined the diminishing concentration of ammonium sulphate necessary to cause commencing precipitation in a series of solutions of increasing protein concentration. Hofmeister in 1888 published the results of similar experiments, using egg-white and potassium acetate and ammonium sulphate. Similar evidence has since been brought forward by other workers, e.g. Mellanby [1907], but in no case was a pure protein employed.

We have made an experiment with pure egg-albumin, estimating the

protein precipitated by a constant concentration of ammonium sulphate (ratio salt to water constant) when the amount of protein was varied. The results are given in Table III and graphically set forth in Fig. 3, where the proportion of the protein separated is plotted as ordinate against the concentration (percentage by weight of whole system) of protein in the original mixture as abscissa. Not only is more protein separated from the more concentrated

TABLE III.

Precipitation of pure egg-albumin with ammonium sulphate; influence of concentration of protein.

Ratio salt/water constant = 31/100.
Concentration of protein varying.

Albumin g.	Water g.	Salt g.	G. albumin in 100 g. total mixture	G. albumin to 31 g. salt and 100 g. H_2O	G. albumin in 100 g. filtrate	G. albumin precipitated from 100 g. total mixture	Protein ppted. %
1·90	56·92	17·65	2·481	3·33	1·130	1·351	54·4
1·90	36·92	11·44	3·775	5·14	1·115	2·660	70·4
3·79	50·85	15·76	5·383	7·45	1·159	4·224	78·5
7·59	53·69	16·64	9·738	14·13	0·935	8·803	90·4
4·74	17·31	5·36	17·306	27·4	0·772	16·534	95·5

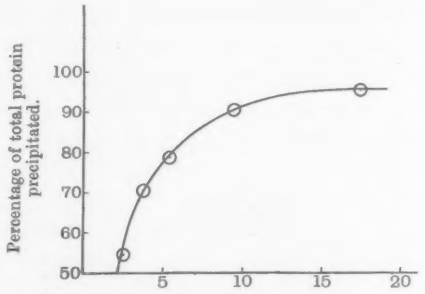

Fig. 3. Influence of concentration of protein upon the precipitation of egg-albumin by $(NH_4)_2SO_4$ at 20° C., see Table III; ratio salt to water constant = 31 to 100, amount of protein varying.

solution at a given concentration of salt, *but a greater proportion is precipitated* (see last column, Table III). The concentration of protein in the filtrate is not constant, but varies from 1·13 % to 0·77 % (col. 6, Table III), as the initial protein concentration is varied from 2·5 to 17·3 % (col. 4). This suggests that the precipitation is a phase separation, analogous to the case of "salting out" of alcohol with ammonium sulphate. As will be

seen later, when the results are given of determinations of the protein-salt-water content of filtrate and precipitate respectively, this was proved to be the case.

3. *Influence of hydrogen ion concentration.*

It is common experience, e.g. with serum, that the addition of a little acid enhances the amount of protein precipitated by the same concentration of ammonium sulphate, and that proteins not precipitated by saturation with sodium chloride are thrown down on acidification of the solution.

Mellanby [1907] called attention to the increased amount of precipitation of horse serum by neutral salts after addition of various acids, and gave some quantitative data, using sodium chloride. In the present investigation the influence of acidity was directly measured in a series of mixtures in which the concentration of protein and ammonium sulphate was maintained constant, and so chosen that precipitation had just begun in the control solution. The reaction of the solution was adjusted to various degrees of hydrogen ion concentration by the addition of small quantities of standard sulphuric acid.

Determinations of hydrogen ion concentration were made with the type of concentration cell described by Michaelis and Rona [1909], the contact fluid between the two cells being saturated potassium chloride solution. It is possible that the determinations of H^+ concentration are not very accurate owing to the high concentration of ammonium sulphate present; those in any one series are, however, perfectly comparable.

TABLE IV.

Precipitation of pure egg-albumin with ammonium sulphate; influence of hydrogen ion concentration.

Experiment I at 18°.
G. ammonium sulphate in 100 cc. original mixture = 30·4.
G. protein in 100 cc. original mixture = 0·575.

G. protein present in 100 cc. filtrate	Hydrogen ion concentration in filtrate, in terms of normality
0·572	$10^{-5.27}$ (54×10^{-7})
0·391	$10^{-5.00}$ ($101 \times$,,)
0·097	$10^{-4.64}$ ($231 \times$,,)
0·062	$10^{-4.53}$ ($296 \times$,,)
0·048	$10^{-4.31}$ ($492 \times$,,)
0·035	$10^{-4.11}$ ($780 \times$,,)

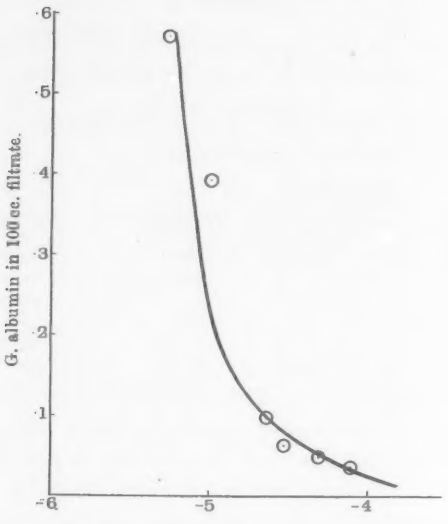

Fig. 4. Influence of hydrogen ion concentration upon the precipitation of egg-albumin by $(NH_4)_2SO_4$ at 18° (see Table IV).

Concentration of $(NH_4)_2SO_4$ in whole system, constant = 30·4 grams per 100 cc.
" " Protein " " " =0·575 " "

The concentration of protein varied from 0·6 to 0·9 % and that of ammonium sulphate from 30·4 to 28·6 % (by volume) in the three different series of experiments set forth in Tables IV to VI. The mixtures were placed for two hours at 18° in order that equilibrium might be attained; they were then filtered and the concentration of protein and of hydrogen ions in the filtrate was determined.

The influence of hydrogen ion concentration was found to be very marked although the range through which it operates is not extensive. In one case (Exp. I, Table IV, protein concentration = 0·57 %, ammonium sulphate = 30·4 %) an increase in acidity from 54×10^{-7} normal (control) to 780×10^{-7} normal was enough to cause precipitation of nearly all the protein. In Exp. II, Table V (0·86 % protein and 30·03 % ammonium sulphate) and Exp. III, Table VI (0·9 % protein and 28·6 % ammonium sulphate) the effective range of hydrogen ion concentration was from 2·9 to 360×10^{-7} normal, and from 12 to 203×10^{-7} normal respectively.

TABLE V.

Precipitation of pure egg-albumin with ammonium sulphate; influence of hydrogen ion concentration.

Experiment II at 18°.
G. ammonium sulphate in 100 g. original mixture = 30·03.
G. protein in 100 g. original mixture = 0·856.

No. of cc. N/10 H_2SO_4 (or equivalent) added in total volume of 27 cc.	No. of cc. N/10 NH_4OH (or equivalent) added in total volume of 27 cc.	G. protein in 100 cc. filtrate	Hydrogen ion concentration (filtrate), in terms of normality
—	1·0	0·864	$10^{-6.54}$ ($2{\cdot}88 \times 10^{-7}$)
—	0·5	0·809	$10^{-6.01}$ ($9{\cdot}82 \times$,,)
—	0·3	0·795	$10^{-5.72}$ ($19{\cdot}0 \times$,,)
—	—	0·514	$10^{-5.40}$ ($39{\cdot}8 \times$,,)
0·5	—	0·056	$10^{-4.98}$ (104 × ,,)
1·0	—	0·01 (about)	$10^{-4.45}$ (358 × ,,)

The last trace of protein present, however, does not appear to be precipitated by alteration of reaction alone, a slight trace in Exp. III being still left in solution at a hydrogen ion concentration of about 1/100 normal or 73000×10^{-7} normal.

The range of reaction where hydrogen ion concentration has its great effect is presumably just on the acid side of the iso-electric point (see below p. 392). From Exp. III it is seen that for ammonium sulphate change in hydrogen ion concentration at and on the alkaline side of the neutral point is without much influence (viz. from $H^+ = 12 \times 10^{-7}$ normal to $0{\cdot}008 \times 10^{-7}$ normal), see Table VI.

TABLE VI.

Precipitation of pure egg-albumin with ammonium sulphate; influence of reaction, hydrogen ion concentration.

Experiment III at 18°.
G. $(NH_4)_2SO_4$ in 100 g. original mixture = 28·6.
G. protein ,, ,, ,, = 0·910.

Cc. N/10 H_2SO_4 (or equivalent) added in total volume of 25·4 cc.	Cc. N/10 $(NH_4)OH$ (or equivalent) added in total volume of 25·4 cc.	G. protein in 100 cc. filtrate	Hydrogen ion concentration (filtrate) in terms of normality
—	3·6	No precipitation	$10^{-9.11}$ ($0{\cdot}008 \times 10^{-7}$)
—	2·0	0·899	$10^{-7.33}$ (0·59 ,,)
—	1·0	0·908	$10^{-6.56}$ (2·7 ,,)
—	—	0·899	$10^{-6.08}$ (12·0 ,,)
0·25	—	0·533	$10^{-5.45}$ (35 ,,)
0·5	—	0·174	$10^{-5.01}$ (97·5 ,,)
1·0	—	0·063	$10^{-4.69}$ (203 ,,)
5·0	—	trace	$10^{-2.45}$ (35000 ,,)
10·0	—	slight trace	$10^{-2.14}$ (73000 ,,)

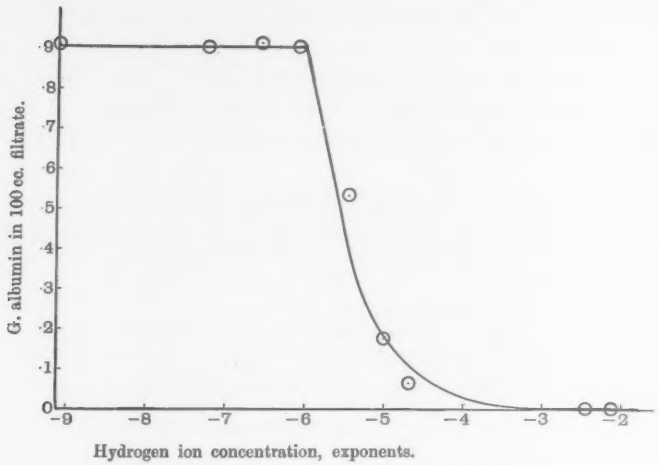

Fig. 5. Influence of hydrogen ion concentration upon the precipitation of egg-albumin by $(NH_4)_2SO_4$ at 18° (see Table VI).

Concentration of $(NH_4)_2SO_4$ in whole system, constant = 28·6 grams in 100 cc.
,, ,, protein ,, ,, ,, = 0·910 ,, ,,
H^+ concentration expressed as exponents.

4. *Influence of temperature.*

Lewith [1888] in case of ox-serum proteins, showed that rise in temperature assisted the precipitation by ammonium sulphate and Hofmeister [1888] made the same observation with egg-white and various salts. Spiro [1904] stated the same to be true of crystalline serum albumin and ammonium sulphate.

We have confirmed the above observations for serum proteins[1] and pure egg-albumin if the reaction be alkaline, but in faintly acid solution (10^{-5} normal) we have found the reverse to be true above 9°.

In Table VII and Fig. 6 are set forth the results of an experiment with 0·85 % protein and 28 % ammonium sulphate. A series of exactly similar solutions were placed for from 1 to 2 hours in a thermostat at the required temperature, after which they were rapidly filtered and the protein estimated in the filtrate. From 0° to 9° the temperature coefficient of precipitation was positive, above 9° it remained negative to 50°[2]. This can be readily

[1] Mellanby [1907, p. 294], on the other hand, states that the temperature coefficient of serum-protein precipitation with $(NH_4)_2SO_4$ is negative between the temperatures of 0° and 40° C. and so small as to be unimportant.

[2] No denaturation occurred.

demonstrated if ammonium sulphate be added to an egg-albumin solution at 9° to the point of opalescence, and just short of the formation of a definite precipitate. If, then, the mixture be divided into three portions, of which one is placed at 0° and a second at 20°, a definite precipitation will occur at both temperatures, whereas the portion maintained at 9° will remain merely opalescent.

TABLE VII.

Influence of temperature upon the precipitation of pure crystalline egg-albumin with ammonium sulphate (28 %).

Protein content = 0·85 %.
Hydrogen ion concentration about 10^{-8} normal.

Temperature	Concentration of protein in filtrate, %
50	0·828
36	0·703
20	0·364
9	0·268
0	0·318

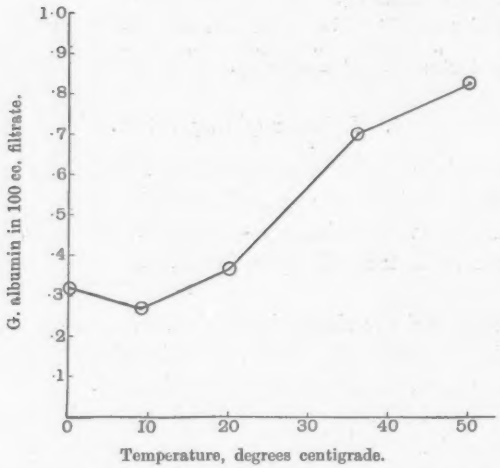

Fig. 6. Influence of temperature upon the precipitation of egg-albumin by $(NH_4)_2SO_4$, see Table VII.

Concentration of protein, salt, water and hydrogen ions in whole system constant.

Interpretation of results.

From the experiments detailed above, it is clear that the amount of protein precipitated from solution (volume of the protein-rich phase) is

dependent upon the amount of salt present, concentration of protein, concentration of hydrogen ions and temperature.

Spiro [1904] showed that, in the "salting out" of caseinogen and gelatin by sodium sulphate, the ratio of salt to water in the more protein-rich phase was less than in the watery phase—a similar relation to that found by de Bruyn [1900] in alcohol separation.

The same is true in the case of egg-albumin. An albumin solution was precipitated by ammonium sulphate and a complete analysis made of the original solution, the precipitate and the filtrate. In order to free the precipitate from adherent mother-liquor, it was not simply dried between filter paper, as done by Spiro, but placed between filter paper, surrounded by kieselguhr and submitted to a pressure of about 3 tons to the square inch. This pressure was, however, more than enough to squeeze out adherent mother-liquor, and actually removed some of the imbibed water of the protein-rich phase, for the concentration of salt in the liquid expressed, in the one case where it was collected and analysed, was only about half of that in the watery phase. The analyses given in Table VIII, where the results of these experiments are set out in detail, do not therefore express the composition of the two phases which were in equilibrium. The experiments prove, however, that the protein had appropriated some of the water for, notwithstanding this squeezing out of weak salt solution, the ratio salt to water in the compressed cake was considerably less than in the watery phase. See Table VIII.

This means that egg-albumin, like caseinogen and gelatin, dissolves or imbibes water just as alcohol does, and a mixture of protein, salt, water can be made to separate into two phases, either by the further addition of salt or of protein in the same way as a mixture of alcohol, salt, water separates on the addition of either salt or alcohol. In either case it amounts to increasing the concentration of salt in the watery phase.

To explain the influence of hydrogen ion concentration in the precipitation of egg-albumin, we must suppose that the electrical condition of the colloidal particles is a factor which modifies the ease with which they aggregate under the influence of the salt present. Hofmeister [1889] was influenced by a similar idea when he made parallel experiments with colloidal ferric hydroxide and egg-white, and Posternak [1901, 1 and 2], when investigating the precipitation of vegetable globulins, found that the efficiency of various electrolytes was influenced by the reaction of the suspension and presumably by the charge originally carried by the protein particles.

TABLE VIII.

Precipitation of pure egg-albumin by ammonium sulphate; composition of filtrate, pressed precipitate and press-liquor.

Exp.	Composition % by weight of	In original mixture	In pressed precipitate	In filtrate	In press liquor
I	Egg-albumin ...	9·56	64·65	0·84	—
	$(NH_4)_2SO_4$	23·35	29·83	26·67	—
	H_2O	67·08	6·39	72·49	—
	Salt present in 100 parts salt and water	25·82	17·6	26·9	—
II	Egg-albumin ...	12·48	63·69	0·59	—
	$(NH_4)_2SO_4$	22·60	29·91	27·04	—
	H_2O	64·92	8·47	72·37	—
	Salt present in 100 parts salt and water	25·82	22·1	27·2	—
III	Egg-albumin ...	9·47	73·57	1·58	0
	$(NH_4)_2SO_4$	24·09	22·04	27·66	15·2
	H_2O	66·42	6·39	70·76	84·8
	Salt present in 100 parts salt and water	26·62	22·47	28·1	15·2

The particles of proteins in acid or alkaline solutions carry respectively a positive or negative electric charge. Only at the iso-electric point, which is slightly to the acid side of the true neutral point in all cases hitherto investigated, does the charge disappear.

Salts like ammonium sulphate and sodium sulphate will cause separation into two phases, one protein-rich and the other protein-poor, whatever the charge upon the protein aggregates of the colloidal solution, if enough of the salt be added. Such phase separation is very materially assisted if the particles are positively charged, i.e. in solutions more acid than the iso-electric point. In the case of sodium chloride, indeed, acidification is necessary with many proteins.

From the analogy of the "salting out" of alcohols, phenols, etc. one must suppose that, by the gradual withdrawal of water from the protein aggregate by the salt, a critical dispersion point is reached when the surface tension at the interfaces causes the particles to run together. Supposing the particles are not iso-electric with the continuous phase, either from the original solution being acid or alkaline, or from the preferential adsorption of one of the ions of the electrolyte, the possession of charge will lower surface tension, so that, with charged particles, a higher concentration of salt will be required to arrive at the critical dispersion point. With negatively charged particles, in an alkaline solution, the charge cannot be neutralised by the more potent

ion SO_4'' which, if adsorbed, would still further increase the negative charge. We find that once the solution is more alkaline than the neutral point the amount of alkali added makes no difference[1] to the amount of salt required for precipitation, see Exp. III, Table VI and curve Fig. 5. The moment the reaction is made more acid than the iso-electric point and the protein particles carry a positive charge, this will at once be neutralised by the adsorption of SO_4'' ions. As these are in such high concentration they are apparently able to counteract in this way the maximum positive charge imposed upon the particles by the addition of acid (see Exp. III, Table VI, $H^+ = 0{\cdot}007$ normal).

We venture to put forward the above interpretation from the analogous action of SO_4'' upon protein particles under conditions which permit of the demonstration of the existence of charge. In acid solution, protein particles, carrying a positive charge, have been shown to be sensitive to the anion of any electrolyte they may encounter. (Hardy [1900] for heated egg-white; Chick and Martin [1912] for denaturated serum proteins and egg-white; Chick [1913] for euglobulin and caseinogen.) Arguing from analogy we may suppose that in the case of egg-albumin the SO_4'' ion of ammonium sulphate will also be more readily adsorbed and any charge on the protein neutralised if the solution be originally on the acid side of the iso-electric point, i.e. if the protein particles carry a positive charge.

The statements detailed above have not actually been substantiated in case of pure egg-albumin, nor has the iso-electric point been determined. This has, however, been done in case of serum-albumin [Michaelis and Mostynski, 1910; Michaelis and Rona, 1910], and these two proteins otherwise display close similarity as regards the conditions of their solution or precipitation. We have not been able to put our interpretation to the direct test because it is impracticable to determine the charge carried by the particles of egg-albumin in the presence of excess of ammonium sulphate, nor under these conditions were we able to study the influence of addition of ions of varying valency. To test our hypothesis we therefore had

[1] In the case of Na_2SO_4, which as regards the influence of acid behaves in an analogous manner to $(NH_4)_2SO_4$, excess of alkali (NaOH) favours precipitation, but, compared with the acid, a high concentration is required. In accordance with the explanation of the influence of reaction set forth above we presume that, while in acid solution the positively charged protein particles attract the SO_4 ion, in alkaline solution the Na ion of the sodium salt is preferentially absorbed. The electric charge on the particles is neutralised in both cases, but more readily in the first, owing to the greater potency of the SO_4 ion. With $(NH_4)_2SO_4$ no effect in solutions made alkaline with ammonia can be demonstrated, owing, presumably, to the low ionisation of $(NH_4)OH$, especially in the presence of excess of $(NH)_2SO_4$.

recourse to the expedient of withdrawing the water from the protein-water-combination by the addition of alcohol to the point where a surface tension is just manifest at the interfaces. In other words, the alcohol was added until the solution became opalescent but short of commencing precipitation, which could then be brought about by addition of a small amount of various electrolytes. Two sets of experiments were made; in one set the original protein solution was acid, and in the second set alkaline, and it was found, as was expected, that an electrolyte was efficient in causing precipitation of the protein in order of increasing valency of its anion in the first case and of its kation in the second. All solutions contained the same concentration of alcohol.

The results of a series of experiments with serum proteins are given in Table IX. In solution A (acid) salts containing divalent (ammonium sulphate) and trivalent (sodium citrate) anions caused precipitation in respective concentrations of 0·00055 and 0·00036 molar, whereas, in case of a monovalent anion (magnesium nitrate) about ten times the concentration was required. The valency of the kation was not without effect, but worked in the opposite direction; a small concentration of lanthanum nitrate (0·0007 molar) cleared up the original opalescence of the solution. For the same reason magnesium sulphate proved to be a much less efficient precipitant than ammonium sulphate.

An exactly converse set of results was obtained when the protein solution was originally made alkaline (B. Table IX). In this case lanthanum nitrate was the most powerful precipitant of all the salts tried and the sulphate of magnesium was much more effective than that of ammonium. At the same time a small concentration of sodium citrate (0·0007 molar) caused the solution to become clear.

With alkaline solutions higher concentration of lanthanum nitrate (0·0036 molar) prevented the formation of a precipitate, no doubt owing to the acquisition of a positive charge in excess of that needed to neutralise the negative one originally possessed. In acid solution an analogous result followed addition of sodium citrate to a concentration of 0·0007 molar.

Some experiments made with pure egg-albumin are set out in Table X. The original solution was acidified and after addition of sufficient alcohol to cause turbidity, determination was made of the concentration of a series of sodium salts necessary to cause a precipitate to form. The influence of increasing valency of the anion is very marked, sodium citrate and sulphate being respectively about 800 and 25 times as powerful in this respect as sodium chloride.

TABLE IX.

Precipitation of serum proteins by various electrolytes in presence of alcohol (almost to precipitation) at 0°.

Protein content = about 0·1 %.

A. In acid solution.

Salt	Conc. (molar)	Conc. (normal)	Degree of precipitation	Conc. (molar) required for complete precipitation
$Na_3\overline{Cit}$	0·00007	0·00022	+	0·00036
	0·00036	0·0011	+ +	
	0·00071	0·0022	+	
	0·0036	0·011	–	
$(NH_4)_2SO_4$	0·00011	0·00022	+ –	0·00055
	0·00055	0·0011	+ +	
	0·0055	0·011	+ +	
	0·055	0·11	+ +	
$MgSO_4$	0·00055	0·0011	+	0·0011
	0·0011	0·0022	+ +	
	0·0055	0·011	+ +	
	0·055	0·11	+ +	
$Mg(NO_3)_2$	0·0011	0·0022	+ –	0·0055
	0·0055	0·011	+ +	
$La(NO_3)_3$	0·00071	0·0022	–	
	0·0036	0·011	–	

B. In alkaline solution.

$La(NO_3)_3$	0·000071	0·00022	+ –	0·00036
	0·00036	0·0011	+ +	
	0·00071	0·0022	+ +	
	0·0036	0·011	+	
$Mg(NO_3)_2$	0·00011	0·00022	+ –	0·00055
	0·00055	0·0011	+ +	
	0·0055	0·011	+ +	
	0·055	0·11	+ +	
$MgSO_4$	0·0011	0·0022	+ +	0·0011
	0·0055	0·011	+ +	
$(NH_4)_2SO_4$	0·0011	0·0022	+ – *	No precipitation
	0·0055	0·011	+ – *	
$Na_3\overline{Cit}$	0·00071	0·0022	–	,, ,,
	0·0036	0·011	–	

* Clearer than control solution containing alcohol only.

– = clear solution, clearer than the control, containing alcohol only.

– + = opalescent solution.

\+ = partial precipitation.

+ + = complete precipitation, filtrate protein-free or containing trace only.

TABLE X.

Precipitation of pure egg-albumin, in acid solution, by sodium salts, in presence of alcohol almost to precipitation; influence of anions.

Protein content = 0·7 %.
Temperature, 0°.

Salt	Concentration required to cause precipitation in presence of alcohol (Molar)	Concentration necessary to commence precipitation in absence of alcohol (Molar)
Citrate (neutral)	0·00013	1·37
Phosphate	0·00016	2·50
Tartrate	0·003	1·60*
Sulphate	0·004	1·69
Acetate	0·09	—
Chloride	0·10	4·3*
Chlorate	0·35	—
Chromate	0·4*	1·71*

* Did not precipitate.

In the same Table are given the results of a series of experiments in which precipitation took place in absence of alcohol and it will be seen that no such influence of valency can be detected here. The relation of the various electrolytes, with the exception of sodium citrate, is that expressed by the Hofmeister series [1888] and doubtless conditioned by their relative water-drawing capacity. When the protein is on the point of being precipitated after the necessary water-withdrawal has taken place, the influence of the anion or kation of the electrolyte present can complete precipitation by neutralising a charge. This occurs for example when protein almost precipitated by ammonium suphate is made slightly acid, or when to protein almost thrown out by alcohol a trace of an appropriate electrolyte is added. In the former case, as long as the solution remains alkaline, the effect of presence of ammonium sulphate with its divalent anion will rather be to increase the negative charge on the protein.

An additional piece of evidence in support of the view that the charge carried by the protein particles is an important factor in determining the concentration of salt requisite to occasion separation into two distinct phases, was obtained from studying the "salting out" of egg-albumin with calcium chloride. In this case the positive ion of the electrolyte is prepotent and we should expect it to act more efficiently if the solution containing the protein is made more alkaline than the iso-electric

point. This proved to be the case[1]. It was found that in a solution containing 0·8 % protein and 37·7 % $CaCl_2$ an opalescence was developed on standing for about 2 hours at 18° when the hydrogen ion concentration was about 23×10^{-7} normal. In presence of a small concentration of $Ca(OH)_2$, under otherwise similar conditions, the hydrogen ion concentration fell to about $0{\cdot}05 \times 10^{-7}$ normal. In this case almost all the protein was precipitated and only a trace remained in the filtrate.

Precipitation by calcium chloride differs from that by ammonium sulphate in the fact that the process is irreversible, and the precipitate is formed slowly and becomes insoluble. We were not able to make satisfactory experiments with other electrolytes of the same character, e.g. the nitrates and chlorides of magnesium and barium, because with egg-albumin precipitation is only very partial even in alkaline solution, but in these cases also, we were able to satisfy ourselves that alkalinity of the solution assisted the separation of a precipitate (protein-rich phase).

Summary.

1. The precipitation of egg-albumin by ammonium sulphate is, as Spiro demonstrated to be the case with sodium sulphate, and caseinogen and gelatin, due to the separation of the system into a protein-rich phase and a watery phase, and to a certain extent is analogous with the salting-out of alcohol.

2. The first effect of concentrated salt is to withdraw water from the protein aggregates. A surface tension is in consequence developed at the interfaces, which causes the protein particles to aggregate, thus dividing the system into two distinct phases (precipitate and filtrate).

3. All three constituents of the system, viz. protein, water and salt, are present in each phase; the proportion, however, is different. The precipitate (protein-rich phase) contains relatively little water and salt, and the filtrate (watery phase) relatively little protein. Under appropriate conditions practically all the protein may be precipitated, only a trace remaining in the filtrate.

The two phases are in equilibrium, and any alteration in the amount of any one of their three constituents is followed by a change both in their composition and volume. Thus increase in concentration of salt or of protein

[1] Precipitation by calcium chloride is also assisted in cases where the solution is markedly acid, a comparatively large concentration of acid (HCl) being needed to demonstrate the phenomenon. The explanation is exactly analogous to that offered for the case of sodium sulphate, see footnote, p. 393, but in the reverse sense, much more acid being required to produce this effect than is the case with alkali.

is followed by a corresponding increase in the protein-rich phase (precipitate). Owing to rigidity of the latter readjustment is, however, slowly accomplished.

4. The relative volume of the two phases (amount of precipitate) is altered by varying the temperature.

5. The exact proportion of salt, protein and water at which phase separation (precipitation) occurs and the relative volume of the two phases (amount of the precipitate) is very sensitive to hydrogen ion concentration in the neighbourhood of the iso-electric point. In the case of $(NH_4)_2SO_4$, when the hydrogen ion concentration varied from 10^{-6} to 10^{-5} normal, the amount of the precipitate increased from a negligible amount to a maximum.

6. With $CaCl_2$, in which the kation is prepotent, a similar effect was observed, but in the opposite direction.

7. An interpretation of the dominating influence of hydrogen ion concentration over this range is put forward and some experiments in support of it adduced.

The principles discussed in this paper must be borne in mind whenever salting-out is made use of in the fractionation of protein solutions and the purification of isolated fractions by re-solution and reprecipitation. Conclusions as to the homogeneity of proteins isolated by salt precipitation must also be reconsidered in the light of these results.

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XL. SOME OBSERVATIONS ON THE ESTIMATION OF UREA.

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The chief aim of the following investigation was to find out a simpler method for the estimation of urea than those at present in use. The hypobromite method, although rapid and simple, has been shown to be unreliable [Mörner, 1903]. All the more accurate methods depend like that of Folin [1901, 1902] on the conversion of urea into an ammonium salt, and the estimation of the ammonia by distillation. These methods necessarily involve a preliminary estimation of the preformed ammonia of the urine. No useful purpose would be served by an enumeration of the chief modifications which the methods have undergone. Reference may be made to the exhaustive account given in the revised edition of Neubauer and Huppert's *Analyse des Harns* [1910].

The distillation of the preformed ammonia and that derived from the hydrolysis of urea occupies in each case about an hour. A considerable reduction of time and simplification of the method would be secured, if the distillation were replaced in both instances by titration in the presence of formaldehyde. The main objections to the latter method are that other amine derivatives, notably amino- and diamino-acids affect the result of the titration [Schiff, 1899, 1901, 1902], and that the best method for the determination of the neutral point, from which one starts, is still unsettled. Ronchese [1907] and Malfatti [1908] employ phenolphthalein throughout as indicator in their method for the estimation of ammonia in the urine by formol-titration, while Sörensen [1907, 1910] and Henriques [1909] use azolitmin paper as the indicator in neutralising the solution and phenolphthalein for the titration of the acids set free by the addition of neutral formol in their method for estimating amino-acids. De Jager [1909, 1; 1910, 1] follows the same procedure as Malfatti after adding excess of neutral potassium oxalate or of potassium sulphate. Folin [1903] had

previously made similar use of potassium oxalate in determining the acidity of the urine. The fact that solutions of chemically pure ammonium salts react acid to phenolphthalein has long been recognised. In consequence, the results of formol-titrations with this as the sole indicator are too low. One objection to the method of overcoming this difficulty adopted by Sörensen and Henriques is the inconvenience of the frequent testing with azolitmin paper required in the course of the titration. The addition of litmus to the solution renders the titration difficult and uncertain owing to the conflicting colour changes of the two indicators. In its place, I have used methyl-red [Rupp and Loose, 1908; Tizard, 1910; Howard and Pope, 1911] as the indicator of the neutral point and phenolphthalein for the determination of the amount of acid set free by the addition of neutral formol. Methyl-red has the advantage of being an exceedingly sensitive indicator for the titration of weak bases such as ammonia, while it is comparatively insensitive to weak acids. On the other hand, phenolphthalein is a good indicator for weak acids, but is not a reliable indicator for weak bases. The colour changes of the two indicators do not interfere with one another, and they may therefore be used together without any difficulty arising. Methyl-orange may be used instead of methyl-red, but it does not give such a sharp endpoint.

1. Preliminary observations on the titration of pure ammonium salts.

The following experiments were carried out in order to ascertain the degree of accuracy of the method as compared with formol-titrations of ammonium salts in the presence of phenolphthalein alone. Five drops of a 0·5 % alcoholic solution of phenolphthalein were added to 10 cc. of a decinormal solution of pure ammonium chloride, which had been previously rendered faintly acid by the addition of dilute hydrochloric acid. The solution was then neutralised by the addition of sufficient decinormal caustic potash just to render the solution faintly red. Excess (about 5 cc.) of neutral formol (50 cc. 40 % formaldehyde, 1 cc. of 0·5 % alcoholic phenolphthalein, and sufficient decinormal caustic potash to render the solution faintly pink) was then added, and the addition of decinormal alkali continued until the solution just acquired a faint permanent red colour. The addition of 9·55 cc. of decinormal alkali was found to be necessary to reach this point instead of the true value 10 cc. Two to three drops of a 0·2 % alcoholic solution of methyl-red were added to another 10 cc. of a decinormal solution of

ammonium chloride, and then sufficient decinormal caustic potash just to convert the red colour of the solution to a yellow. On the addition of neutral formol, the solution again acquired a red colour owing to the hydrochloric acid, set free by the interaction of the formaldehyde with the ammonium chloride, acting upon the methyl-red. On the further gradual addition of a measured quantity of decinormal caustic potash, the red colour fades and is replaced by a yellow. The addition of alkali was continued until a faint permanent red colour, due to the action of the alkali on the phenolphthalein, just appeared. 9·95–10 cc. were required to reach this point. Repeated control estimations of the same character with solutions of pure ammonium sulphate as well as of ammonium chloride gave similar results. It will be noted that, when the two indicators are used, the solution during titration passes through three changes of colour. It is first red owing to the action of free mineral acid on the methyl-red, then orange to yellow when the solution contains only free organic acid, and finally red when just alkaline owing to the action of slight excess of free alkali on the phenolphthalein. The organic acid is formic acid present in the formaldehyde solution and resulting from the oxidation of part of the formaldehyde. Thus, prior to the addition of the neutral formol, the solution contains only ammonium chloride; while after its addition the fluid contains hexamethylenetetramine, free hydrochloric and formic acids, the latter two being in varying proportions according to the amount of formic acid present in the original solution of formaldehyde. In the presence of traces of free formic acid, methyl-red has nearly the same tint as in neutral solution, and, therefore, when all the free mineral acid has been neutralised, the colour of the methyl-red changes to faint orange and later to yellow, although the solution is found still to be distinctly acid to phenolphthalein.

2. Application of the foregoing results to the estimation of pure urea in aqueous solution.

Folin's method of hydrolysis [1901, 1902] is not applicable in this case, since the presence of a large amount of magnesium chloride interferes with the subsequent titration. A slight modification of Benedict and Gephart's method of hydrolysis [1909] was consequently adopted. The modification consisted merely in using more dilute acid for the hydrolysis of the urea. When a considerable excess of acid is used, the large amount of neutral salt formed in the course of neutralisation interferes with the accuracy of the determination of the endpoint [Michaelis and Rona, 1909; Andersen, 1911].

The use of a smaller amount of acid also lessens the risk of the decomposition of other nitrogenous substances, when the method is applied to urine. After using this method of hydrolysis for some time, I found that it had been previously employed by Henriques and Gammeltoft [1911].

At first the hydrolysis was carried out in sealed glass tubes heated in a glycerol bath to 160° for four hours. Two cc. of a 2·006 % solution of pure urea and 2 cc. of normal sulphuric acid were placed in a glass tube, which was then sealed and heated in a glycerol bath. After being allowed to cool, the sealed tube was opened and the contents carefully transferred to a flask, the broken tube being thoroughly washed with distilled water. The fluid was then neutralised in the presence of methyl-red as indicator, and the ammonia determined by means of formol-titration using phenolphthalein as the indicator. The following are the results given in tabular form. One cc. of decinormal alkali is equivalent to 0·003 g. of urea.

Cc. of 2·006 % urea	Cc. of N. acid for hydrolysis	Cc. of N/10 alkali (formol titr.)	G. of urea found	Theoretical amount of urea
2	2 H_2SO_4	13·3–13·35	0·0399–0·04	0·0401
2	2·5 HCl	13·4	0·0402	0·0401

Since the use of sealed tubes is somewhat inconvenient, Benedict and Gephart's method [1909] of heating the solution in test-tubes covered with leadfoil and placed in an autoclave kept at a temperature of 155° for 1·5 hours was afterwards employed. A large number of estimations can readily be carried out simultaneously by means of this method. The temperature in the autoclave was varied in the first experiments in order to find the most suitable temperature for hydrolysis.

2·3101 g. of pure urea were placed in a 200 cc. flask, dissolved in water, 100 cc. of normal sulphuric acid were added, and the whole made up to 200 cc.; 5 cc. portions of the acid solution were then hydrolysed. The following table gives the results.

Temperature	Cc. of N/10 alkali for formol-titr.	G. of urea found	Theoretical g. of urea
144–145° (70 minutes)	18·8	0·0564	0·0577
152–153°	(1) 19·15	0·05745	,,
(30 minutes	(2) 19·2	0·0576	,,
longer)	(3) 19·15	0·05745	,,

Since the application of the foregoing results to the estimation of the urea in the urine involves a preliminary formol-titration of preformed ammonia and other amine derivatives, it was essential to test the method by applying it to the titration of amino-acids as well as to mixtures of amino-acids with ammonium salts.

3. Titration of amino-acids alone and with ammonium salts.

Glycine being easily prepared in a pure state, was selected as a type for the tests. Since one has to deal with a mineral acid solution in the final formol titration, it was obviously essential to carry out the estimation under similar conditions, and also to compare the results with those obtained when phenolphthalein was used as the sole indicator. The following are the results given in tabular form. The glycine solution was 0·71 %.

These results indicate that, when the two indicators are used, the estimations of amino-acids and ammonium salts are more accurate than when phenolphthalein is the sole indicator. The fact that formol-titration yields too low results in the case of mixtures of amino-acids and ammonium salts was first noted by de Jager [1909, 2]. Henriques and Sörensen [1910] have suggested an explanation of this anomaly. More recently de Jager [1910, 2] has shown that the addition of urea enables one to obtain correct results. The latter fact is illustrated by the results given in (4) of the table.

	Solutions used	Cc. N/10 alkali (for neutralisation)	Formol-titr. (found)	Theoretical result
(1)	10 cc. glycine 2·55 cc. N/10 HCl	2·55 (indicator methyl-red)	9·4	9·46
(2)	10 cc. glycine 2·75 cc. N/10 HCl	2·85–2·9 (indicator phenol-phthalein)	9·25	,,
(3)	10 cc. glycine 10 cc. N/10 NH_4Cl 2 cc. N/10 HCl	2 cc. (methyl-red)	18·65	19·46
(4)	10 cc. glycine 10 cc. N/10 NH_4Cl 2 cc. N/10 HCl 0·5 g. urea	2 cc. (methyl-red)	19·4	,,

4. Estimation of urea in urine.

(1) *Without a preliminary purification of the urea.*

Since the presence of phosphates renders the neutral point indefinite for both indicators, and since the neutral points for the two indicators are markedly different in solutions of phosphates, it was necessary to precipitate the phosphates with baryta mixture. The addition of an unduly large excess of barium chloride is to be avoided, as its presence interferes slightly with the accuracy of the titration; but a small excess is immaterial. When sulphuric acid is used for the hydrolysis of the urea the excess of barium present in the filtrate is precipitated as barium sulphate, and it

was mainly for this reason that normal sulphuric acid rather than hydrochloric acid was used for the majority of the hydrolyses. The results of the method of formol-titration were controlled by comparison with the values for ammonia obtained by distillation with magnesia and with barium hydrate.

100 cc. of normal urine were mixed with 10 cc. of a binormal solution of barium chloride and sufficient barium hydrate to make the mixture distinctly alkaline, and finally made up with water to 200 cc. After the fluid had been thoroughly shaken, it was allowed to stand either for a few minutes or preferably in the presence of toluene for twelve hours. Henriques' method [1909] of freeing the urine from phosphates, sulphates and carbonates may be used as an alternative to the foregoing. The solution was then filtered. 25 cc. of the filtrate required 18·5 cc. of decinormal hydrochloric acid to render it neutral to methyl-red. The endpoint is not quite sharp. Excess of neutral formol (about 5 cc.) was added, and the titration with decinormal alkali completed. The average of two titrations was 5·2 cc. of decinormal alkali. This result gives the approximate amount of nitrogen in the form of preformed ammonia and amino-acids in 12·5 cc. of urine.

A number of 10 cc. portions of the filtrate were then mixed with 7–8 cc. of normal sulphuric acid, and placed in test-tubes in the autoclave, which was kept at a temperature of 155° for 1·5 hours. Although the fluid had darkened considerably in colour as a result of the heating with acid, the colour on dilution was not so deep as to interfere appreciably with the subsequent formol-titration. The ammonia in some portions was then set free by the addition of magnesia or barium hydrate, distilled off, and absorbed by excess of decinormal sulphuric acid (50–100 cc.) placed in the receiver. In others the ammonia and amino-acids were determined by formol-titration. Prior to carrying out the formol-titration it is advisable although not essential to filter off the barium sulphate. A considerable amount of pigment adheres to this and is removed with it. The test-tube and precipitate were then thoroughly washed, and the collected filtrate and washings treated by formol-titration. The following table (I) gives the results obtained. The deduction referred to in the table corresponds with the amount of substances in the original urine capable of titration by the formol method, consisting mainly of preformed ammonia and amino-acids.

An examination of the table indicates that the results of the formol-titration are invariably higher than those obtained by distillation; but agree well with one another. The difference is to be explained by the presence of non-volatile amino-groups set free by the hydrolysis with acid,

which raise the results obtained by formol-titration; but obviously cannot affect the values for ammonia obtained by distillation. The hydrolysis with normal acid splits up hippuric acid and any polypeptides which may be present, and thus renders the results of formol-titration higher than those obtained by distillation. The preliminary formol-titration, on which the deduction is based, gives the amount of nitrogen in the form of preformed ammonia and amino-acids: while, after hydrolysis, the final formol-titration gives the preformed ammonia and amino-acids, together with the amino-acids set free by hydrolysis, and the ammonia resulting from the decomposition of the urea. The difference between these two values represents the ammonia derived from urea plus the nitrogen of any amino-acids derived from hydrolysis of polypeptides and of hippuric acid. On the other hand, the method of distillation gives only the preformed ammonia and that formed by hydrolysis of urea. In calculating the results given in the table, the same deduction was made from the values obtained by distillation as from those by formol-titration. Strictly speaking, a smaller deduction should have been made from the latter. An independent determination of the preformed ammonia would be required to ascertain the true value of this correction. This point will be dealt with in a later part of this paper.

TABLE I.

(The quantity of urine was 5 cc. for each estimation.)

Method	Cc. of N/10 alkali	Deduction	% of urea	% of urea-N.
Formol-titration	(1) 32·15	2·1 cc. N/10 alkali	1·803	0·8414
,,	(2) 32·1	2·1	1·800	0·840
,,	(3) 32·4	2·1	1·818	0·848
Distillation with barium hydrate	(1) 32·05	2·1	1·797	0·8386
,,	(2) 32	2·1	1·794	0·837
,,	(3) 31·9	2·1	1·788	0·834
Distillation with MgO	(1) 31·7	2·1	1·776	0·829
,, ,,	(2) 31·75	2·1	1·779	0·830

The difference between the result obtained by distillation and that by formol-titration gives an approximate value for the non-volatile amino-derivatives. A more accurate estimate might be made by taking advantage of the fact ascertained by de Jager [1910, 2] that the addition of urea is necessary in order to secure the true result for the sum of amino-acids and ammonia. This addition is unnecessary in the first formol-titration, since sufficient urea is present in the urine; but after hydrolysis the addition

of pure urea to the neutralised solution would be necessary before carrying out the second formol-titration. I have not considered the correction of sufficient importance in the case of the normal urines examined to warrant its introduction into the table; but in the event of a marked difference being found between the values obtained by distillation and formol-titration indicating excess of amino-acids, it would be advisable to make the correction.

The next table (II) gives the results of a similar series of estimations in a sample of another urine. Normal hydrochloric acid was used in this instance instead of sulphuric.

The same features will be noted as in the previous table. When barium hydrate is used as the fixed alkali, the values for ammonia are usually higher than when magnesia is employed. In order to obtain constant results, the distillation has to be more prolonged in the case of magnesia than with barium hydrate. The distillation usually occupied 1–2 hours in both cases. The differences between the results of formol-titration and of distillation is greater than in the other urine.

TABLE II.

(The quantity of urine used for each estimation was 5 cc.)

Method	Cc. of N/10 alkali	Deduction	% of urea	% of urea-N.
Formol-titration	(1) 36·65	2·5	2·049	0·9562
,,	(2) 36·6	,,	2·046	0·9548
,,	(3) 36·6	,,	2·046	0·9548
Distillation with barium hydrate	(1) 35·45	2·5	1·977	0·9226
,,	(2) 35·65	,,	1·989	0·9282
,,	(3) 35·75	,,	1·995	0·931
Distillation with MgO	(1) 35	2·5	1·95	0·910
,, ,,	(2) 35·5	,,	1·98	0·924

(2) *Estimation of urea in urine after a preliminary extraction of the urea with a mixture of alcohol and ether.*

Folin's [1901] and Benedict and Gephart's methods [1909], as well as all other methods depending on the conversion of urea into the ammonium salt of the acid used for hydrolysis, give inaccurate results when carbohydrates are present in the urine. Mörner [1903] has shown that this is due to the fact that the humin-substances formed by the action of the acid on the sugar contain firmly bound nitrogen, when ammonium salts are present or formed during the hydrolysis.

Mörner adopted the following method in order to obtain a solution of urea free from carbohydrates. The phosphates were precipitated from 5 cc. of urine by the addition of 1·5–2 g. finely powdered barium hydrate, and the fluid was then extracted with 100 cc. of a mixture of two volumes of alcohol and one of ether. After being thoroughly mixed, the solution was allowed to stand for 24 hours, then filtered, and the precipitate washed with the mixture of alcohol and ether, the filtrate and washings being collected in the flask afterwards to be employed in the distillation. The alcohol and ether were then distilled off, and the residue after being freed from ammonia by concentration at 50° after the addition of magnesia, was hydrolysed by Folin's method [1901]. I carried out the first stages of the method in the same way; but instead of freeing the aqueous solution from ammonia by the addition of magnesia, I determined the amount of amino-nitrogen by means of formol-titration. The method of hydrolysis adopted was the modification of Benedict and Gephart's method [1909] previously described. In some cases normal hydrochloric acid was used for the hydrolysis, in others normal sulphuric acid. The following table summarises the results.

TABLE III.

Method	Results in cc. of N/10 NH_3	Deduction	% urea	% urea-N.
1. Direct formol-titration	(*a*) 29·55	0·85	1·722	0·8036
	(*b*) 29·7	,,	1·731	0·8078
	(*c*) 29·65	,,	1·728	0·8064
2. Distillation with $Ba(OH)_2$	(*a*) 29·1	0·85	1·695	0·791
	(*b*) 29·15	,,	1·698	0·7924
3. Distillation with MgO	(*a*) 28·45	0·85	1·656	0·773
	(*b*) 28·5	,,	1·659	0·774
	(*c*) 28·1	,,	1·635	0·763

The following are the chief features revealed by an examination of the table. The comparatively low value of the deduction is to be explained by the fact that the method of preparing the aqueous solution of urea involves the loss of the greater part of the preformed ammonia. The values for urea obtained by the formol method are, as in the previous estimations, higher than those obtained by distillation. Amino-derivatives evidently pass into the alcohol and ether mixture. Hippuric acid is probably the most important of these. The glycine derived from its hydrolysis must raise the results of the formol-titration, while it obviously cannot affect the values obtained by distillation. The results obtained by distillation with magnesia are considerably lower than those with barium hydrate.

5. Final control of the method by an independent determination of the preformed ammonia.

The estimations hitherto given suffer from the defect of not having been controlled by an independent determination of the preformed ammonia. Reference was previously made to this fact. In a final series of experiments, I have estimated the total nitrogen by Kjeldahl's method, the nitrogen of the preformed ammonia by the method of Krüger, Reich, and Schittenhelm [1903], the ammonia and urea nitrogen after hydrolysis by distillation, and the nitrogen of the amino-acids by Henriques' method [1909]. The results for 100 cc. of urine are stated in Table IV in terms of decinormal alkali and in Table V as percentages.

TABLE IV.

Results for 100 *cc. urine expressed in terms of decinormal nitrogen.*

Total N.	Urea and NH_3-N. (distillation)	Urea + NH_3 + amino-acid nitrogen (formol-titration)	NH_3 + amino-N. (in original urine)	Ammonia-N. (distillation)
587	529	550	33·6 (Sörensen)	23·9
585	528	544	35·2 (methyl-red)	24·2
583	524	546	—	—
585 (mean)	527 (mean)	547 (mean)	—	24·05 (mean)

TABLE V.

Total N.	Urea-N.	Urea-N. (formol-titr.)	Amino-N.	Ammonia-N.
585	503	513 (Sörensen)	9·6 (Sörensen)	24·05
—	—	511·4 (methyl-red)	11·2 (methyl-red)	—
0·819 %	0·704 %	0·718 % (S.) 0·716 % (M.)	0·013 % (S.) 0·0157 % (M.)	0·0337 %

The value given in column 2 of Table V is arrived at by deducting the amount of preformed ammonia as determined by the method of Krüger, Reich, and Schittenhelm from the total amount of ammonia obtained by distillation after complete hydrolysis of the urea. This may be regarded as the correct value for urea arrived at by a standard method. The values for the nitrogen of urea given in the third column of Table V are obtained by deducting the results of column 4, Table IV, from those of column 3, Table IV, using in the first instance the results of Henriques and Sörensen's method and in the second case those obtained by the use of methyl-red and phenol-phthalein as the two indicators. The values for the nitrogen of the amino-acids given in column 4 of Table V are arrived at by subtracting the

results of the method of Krüger, Reich and Schittenhelm given in column 5 of Table IV from those of column 4, Table IV, using in the one case Henriques' method, and in the second formol-titration with methyl-red as the first indicator.

Sörensen [1907, 1910] used fifth-normal alkali for the titration of the amino-acids. Notwithstanding the fact that this strength is to be preferred for reasons stated by that author, I was obliged to continue the use of decinormal alkali as it had been used throughout the foregoing investigation. These final results indicate that the error in the method of estimating urea in this urine by formol-titration is a positive one amounting to approximately 1·7 %.

The chief advantages of the formol-method of estimating urea are that it is not only more rapidly carried out than the methods involving distillation, but also entails less arrangement of apparatus. The fact that the method gives at the same time an approximate estimate of the preformed ammonia and amino-acids is also an advantage, which compensates to some extent for the less degree of accuracy attainable. These facts suggest that it might find useful application for the examination of urines in clinical laboratories as a substitute for the hypobromite method. Another possible application of the method might be to the study of the hydrolysis of urea by enzymes and micro-organisms.

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XLI. GAS-ELECTRODE FOR GENERAL USE.

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The standard forms of hydrogen-electrode, such as those used by Dolezalek [1899], Wilsmore [1900] and Bjerrum [1910] and, for fluids containing carbon dioxide, Hasselbalch [1910], must remain indispensable when physical measurements of the highest order of accuracy are undertaken.

With these, after the expenditure of considerable time and care, and with continued vigilance over every part of the electrical apparatus, consecutive results concordant to about 0·05 millivolt can sometimes be obtained. But, for these results to have any meaning beyond the nearest millivolt or two, it is essential that the diffusion potential difference of the cell should be accurately known in every determination: and as this is recognised, by Cumming and others whose investigations continue to throw light on this difficult subject, to be almost impossible, it follows that, as far as absolute $H^{\cdot}$ ion determinations are concerned, attention should be focussed more particularly on diffusion potential errors (in view of their greater magnitude) than on those found at the electrode itself.

In general laboratory practice, therefore, especially when dealing with protein-containing materials, which may or may not be free from carbon dioxide, the most suitable electrode is not one which, when coupled with an ideal electrical apparatus, will give results of this high order of accuracy, regardless of the expenditure of time and material. Rather it is one which, without exaggerating appreciably the diffusion potential error, will give, under ordinary working conditions and in a few minutes, results correct to 1 millivolt or so on one or two cc. of fluid without loss or contamination.

Anticipating a long series of $H^{\cdot}$ ion determinations on protein solutions, some of them containing carbon dioxide, I endeavoured to devise such an electrode. The arrangement subsequently adopted and the experiments made to discover its imperfections are described below. Only one form of

apparatus has been used for all cases. When dealing with a solution containing carbon dioxide or other dissolved gases a slight modification of technique is all that is necessary.

Apparatus.

The modifications of existing apparatus which I have employed consist of

(1) the electrode vessel,
(2) the filling apparatus,
(3) the support in the constant temperature bath.

The electrode vessel (*ABD*, Fig. 1) is somewhat more complicated than the simple V shape generally employed, but this is amply compensated for by the simplicity of the other parts of the apparatus and the many conveniences accompanying its use. I have used throughout the platinum point advocated by Michaelis, just making contact with the surface of the fluid. The platinum point is mounted at the end of a glass tube *A*. The protruding end is blackened in the usual manner. The other end makes contact with a small globule of mercury placed inside the tube.

The lower end of *A* may be ground to fit the outer tube *B* or else a joint may be made by means of a rubber stopper as in the diagram. Attached to *B* at the side is a very fine bore capillary tube carrying a tap *D*, and at the lower end is a second capillary tube not quite so fine, of about 1 mm. bore. At the lower end of this may be fitted a very small glass stopper, but it is not necessary and has certain disadvantages.

The filling apparatus used with all these electrode vessels is depicted together with other apparatus in Fig. 2. It consists of a three-way piece *F* connecting a 5 cc all-glass syringe *G*, well lubricated with vaseline, a short piece of fine-bore stout rubber tubing *H* and a glass tube carrying a glass stopcock which is connected with the hydrogen supply.

As *support* for the electrode vessels in the constant temperature bath, while taking potentiometer readings, I have used a glass trough *K* (Fig. 1) in which the electrode vessels stand side by side in a suitable connecting solution. For single electrode vessels a large test tube serves excellently. The other half-electrode employed, a tube from which leads into the glass trough, is also immersed in the water-bath.

I have used principally the calomel-saturated potassium chloride half-electrode recently described by Michaelis and Davidhoff [1912] and a standard calomel N/10 KCl half-electrode. The connecting solutions were saturated potassium chloride solution, saturated ammonium nitrate [Cumming, 1907],

and solutions of potassium chloride 1·75 N and 3·5 N respectively, employed for the extrapolation method of Bjerrum.

In some experiments on diffusion potential two gas electrodes of the vertical type were stood side by side in the trough containing connecting solution.

Experiments with solutions free from carbon dioxide and other dissolved gases.

Technique. The electrode vessel is first of all connected by the capillary side tube *D* (Fig. 1) to the rubber tube of the filling apparatus (shown at *GFH*, Fig. 2), and hydrogen passed for a few seconds, both glass taps being open. Then, by alternately drawing out the piston with the tap on the electrode vessel shut, and pushing it home with the tap open, the last traces of air are expelled from the dead space of the syringe and the T-piece. The lower end of the electrode vessel is now brought under the surface of a sample of the fluid to be examined and the glass tap on the T-piece shut. By pulling out the plunger of the syringe the fluid is drawn up until its surface is just at the point where the platinum point is sealed to the glass. Slight movements of the plunger in and out now cause the liquid by rising and falling to wash the platinum point well without wetting the tube *A* (Fig. 1) supporting it. Finally the height of the liquid is adjusted till the point just touches the surface, the glass tap *D* on the side capillary closed, and the vessel containing the remainder of the sample of fluid taken away.

The electrode vessel is now disconnected from the filling apparatus, and the lower capillary wiped dry with a piece of clean filter-paper and placed in the glass trough in a constant temperature bath. There is sufficient connecting solution in the trough to cover the electrode vessel above the rubber stopper. The two copper wires from the potentiometer are now led, one into the calomel half-electrode, and the other into the tube *A* and the reading is taken from which the value of P_H^+ (the negative exponent of the H^+ ion concentration) may be calculated directly.

In order to be certain that the platinum electrode is working properly and that equilibrium is established instantaneously, a few minutes at least may be allowed to elapse before a second reading is taken. Meanwhile the filling apparatus can be used to fill another electrode vessel with another sample, and when this is introduced into the trough, the copper wire can be changed over from the first electrode vessel and a reading of that taken. While the equilibrium of the second electrode is being checked, the first can

be refilled. It is removed, dipped into a large beaker of distilled water, and dried externally with a clean towel or filter paper and connected again to the filling apparatus.

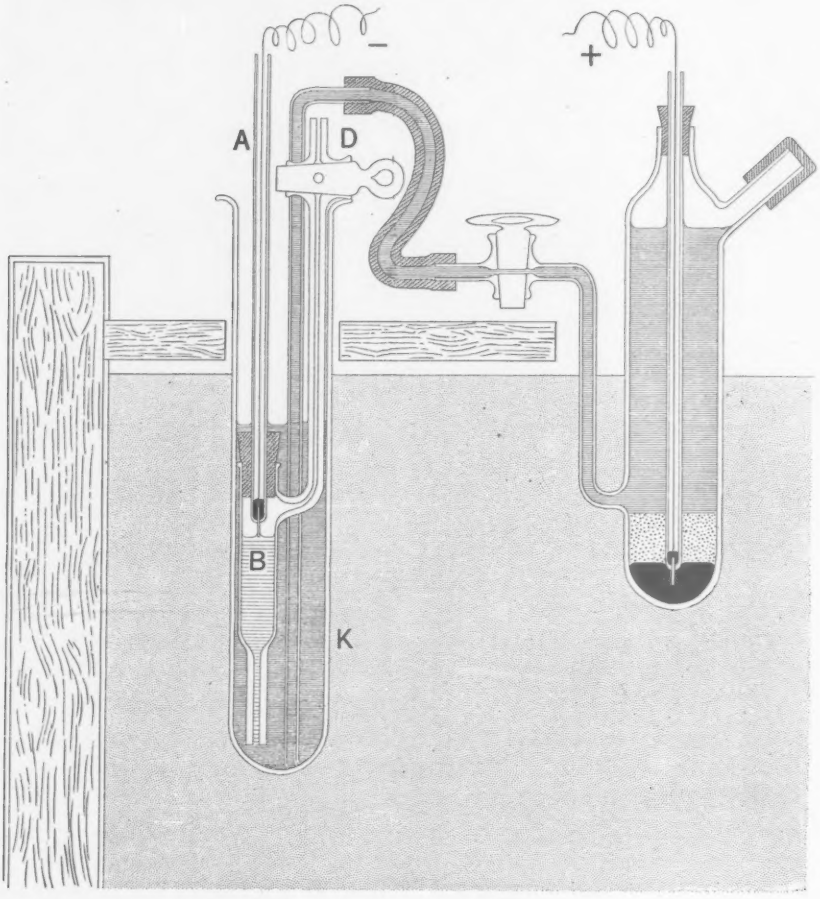

Fig. 1.

Opening the tap *D* (Fig. 1) and pushing home the syringe, the electrode vessel is emptied, and the tap on the filling apparatus opened for a few seconds to fill it with fresh hydrogen. The next sample having been brought underneath the electrode vessel, the tap on the three-way piece is closed,

liquid drawn up by the syringe, expelled so as to get rid of traces of the previous sample, and then the vessel filled with a fresh sample as before.

If an electrode is properly blacked, and in good working order, it should give the equilibrium value immediately and scarcely change afterwards—two millivolts at most—during the next ten or twenty minutes. Diffusion of hydrogen into and through the rubber stopper causes slight inconvenience if the electrode has not been used for a day or two. The liquid rises in the vessel and the platinum becomes immersed beyond the point. Equilibrium under these conditions would not be reached for a long time. To rectify this, the glass tube *A* can be slid upwards through the rubber stopper. It is advisable when setting the electrode aside, to push the glass tube holding the electrode well through the cork, fill with hydrogen, suck up distilled water till the platinum is immersed and after disconnection from the filling apparatus leave the vessel immersed in distilled water.

Washing out of electrode vessel. One rinsing only between two samples has been found sufficient. Several experiments were performed to demonstrate this. One set of figures is given below.

Temp. 18°. Bar. 750 mm. Half-electrode sat. KCl-calomel. Connecting fluid sat. KCl. Calculated P_H^+ value for 0·0993 N HCl (dissociation 91·6 %) = 1·041. Calculated P_H^+ value 0·1 N NaOH (14·14 − 1·075) = 13·065. Sample used was known not to be absolutely free from CO_2.

Solution used	Volume used	Remarks	Time of observation	Potential π	$P_H^+ = \frac{\pi - 0{\cdot}251}{0{\cdot}0577}$
0·0993 N HCl	2 cc.	Apparatus at commencement clean and dry	8.45	0·3110	1·039
			8.50	0·3110	1·039
			9.10	0·3113	1·045
,,	4 cc.	Used 2 cc. for rinsing out last sample	1.45	0·3113	1·045
			2.00	0·3115	1·048
			2.15	0·3120	1·057
			2.45	0·3120	1·057
0·1 N NaOH	4 cc.	Used 2 cc. for rinsing out last sample	2.50	1·0020	13·015
			2.55	1·0022	13·019
			3.15	1·0022	13·019
			3.50	1·0030	13·032
0·0993 HCl	4 cc.	Used 2 cc. for rinsing out last sample	4.19	0·3115	1·048
,,	circ. 20 cc.	Rinsed out many times and filled side capillary, see page 425. Passed bubble for 5 mins.	4.45	0·3105	1·031
			4.55	0·3110	1·039
			5.00	0·3110	1·039
			next morning 8.5	0·3108	1·036

Temperature. Although most of these determinations have been made at 18° exactly, it is unnecessary to pay too great attention to this point as long as the temperature of the calomel-saturated KCl half-electrode and the gas

electrode are the same in any one determination. Calculation from the constants given by Michaelis for this combination demonstrates that the error in measurement of the value of P_H^+ per 1° is 0·007 for a neutral solution the H· ion concentration of which has no temperature coefficient. The error with a N/10 KCl-calomel electrode is about three times this. Approximate determinations with the saturated KCl half-electrode may quite well be made on the open bench without a constant temperature bath.

When a N/10 KCl-calomel half-electrode or a N/10 HCl-hydrogen half-electrode are used in combination with the gas electrode, more careful temperature adjustment is necessary. As a direct result of the diminutive size of the electrode vessel, and the small quantity of fluid used therein, the time taken to arrive at temperature equilibrium is very short.

Quantity of material used. The size of electrode vessel found most convenient for general use has the following principal dimensions; length of tube *B* (Fig. 1) 75 mm., maximum external diameter 10·5 mm., length of lower capillary 25 mm. The capacity when full is 1·5 cc., so that a determination can be performed easily on 2 cc. of the material if the electrode vessel be clean and dry to start with. When the electrode vessel is not cleaned and dried between two determinations, 4 cc. are required—2 cc. to rinse out the vessel and 2 cc. from which the electrode vessel is filled.

If only smaller quantities of material are available, a smaller electrode vessel may be used. Quite good results were obtained by one holding 0·3 cc. This is not necessary, however, for the quantity of material available may be drawn up into a vessel of the most convenient size and then saturated potassium chloride drawn up afterwards until contact is made with the platinum point. Mixing of the two fluids only takes place very slowly by diffusion unless the density of the fluid examined approximates to that of saturated KCl. In those cases when it is known that the H· ion concentration does not change by such treatment the sample may be diluted with pure water [cf. Michaelis and Davidhoff, 1912]. This is the substitution of an indirect method for a direct one and so an additional possibility of error is introduced.

The extent of the contamination of the fluid during a determination is very small since the boundary surface between it and the saturated potassium chloride solution is only that of a section of the capillary tube. When a very small glass stopper is fitted at the bottom of the capillary, possibility of contamination is still further removed. It is usually found that enough potassium chloride diffuses round the stopper to diminish the resistance sufficiently for a reading, not always very sharp, to be taken.

In many cases, therefore, where a small quantity of liquid only is available, it is possible to determine its reaction electrometrically and recover it with very little contamination, dilution, or loss, for further experiments. Usually a moderate economy of material is advantageous and, on this and other grounds, the use of the vertical electrode filled by suction has been found by me much more convenient than the V-type for general work. Frothing fluids do not exhibit their disagreeable characteristic, as hydrogen is not bubbled through them, but they are forced up by external atmospheric pressure. There is no need, therefore, to make an indefinite contact with the froth. The abolition of tapes or wool threads soaked in potassium chloride solution was found a great convenience.

Diffusion Potential. In the electrolytic cells of which one half-electrode is hydrogen in contact with an aqueous solution there are really four differences of potential involved. There are the two electrode potentials proper and two differences of potential where the electrode solutions come into contact with the connecting solution. The latter differences of potential are for the most part dependent upon the natures of the electrode solutions and of the connecting solution, but the time the solutions have been in contact is a concomitant factor. Cumming and Gilchrist [1913] have recently investigated this quantitatively and find that this "time change" is more marked with capillaries than open tubes. They, therefore, recommend that capillaries should be avoided in the construction of an electrolytic cell; so also should membranes, cotton wool plugs and, presumably, tapes.

In the vertical electrode vessel filled by suction, the surface between the solution examined and the connecting fluid is in a capillary or at the end of one. It may readily be made to occur at the wider part of the tube (p. 415, paragraph 4). I have made a number of careful measurements with this cell to determine how far variations in potential difference can be traced to boundary changes, and to what extent these alter when the surface between the electrode solution and the connecting solution is in the broader part of the tube instead of at the end of the capillary.

Using N HCl and N/10 HCl as electrode solutions connected to a saturated KCl-calomel half-electrode by saturated KCl solution, I could not detect with certainty any difference in the time change over half an hour in each case—first with boundary at end of capillary, second with boundary in broader part of the tube. Over longer periods, differences in the "time changes" could probably be detected, but do not rightly enter into consideration in the ordinary use of the electrode.

I have sometimes used saturated ammonium nitrate [Cumming, 1907]

instead of saturated potassium chloride as connecting fluid when examining acid solutions. With the N/10 KCl-calomel half-electrode N and N/10 HCl gave the potentials 0·3390 and 0·3974 respectively; calculated values 0·3407, 0·3977. With the saturated KCl-calomel electrode, however, the results were about 18 millivolts too low, the contact potential between saturated potassium chloride solution and saturated ammonium nitrate being of that order.

Comparison between the results obtained with the vertical and the V-electrode vessels.

Both electrode vessels were fitted with rubber stoppers through which passed the glass tube in the end of which the blackened platinum point was mounted. When necessary this was adjusted so as just to touch the surface of the liquid. Temp. 18°. Connecting solution saturated KCl solution.

Date	Solution	Half-electrode	Potential readings: V-electrode vessel	Potential readings: Vertical electrode vessel	Previous results
18 April	0·01 Na_2CO_3 Aq.	Calomel. Saturated KCl	0·8770	0·8780	—
11 April	Sodium Citrate (Sörensen)	,, ,, ,,	0·6240	0·6235	0·6238 (Sörensen)
6 May	Standard Acetate solution (Michaelis)	,, ,, ,,	0·5165	0·5170	0·5175 (Michaelis)
7 May	0·1 N HCl	,, ,, ,,	0·3122	0·3125	—
8 May	0·1 N HCl	,, ,, ,,	0·3122	0·3125	—
15 May	N HCl	,, 0·1 N KCl	0·3435	0·3425	—
15 May	N HCl	,, Saturated KCl	0·2558	0·2560	—

Electrometric Titration.

In acidimetry and its various applications the addition of a standard alkaline solution is continued until the titrated fluid has a certain $H^{\cdot}$ concentration previously agreed upon. This is called the "end point." Generally the "titre" or amount of standard alkali which must be added to a certain amount of the fluid to be titrated in order to arrive at the "end point" is all the information required. The observer, with a proper understanding of the nature of the reaction, knows the correct "end point" for the object he has in view, and chooses an indicator which will tell him by its colour change when the "end point" is reached.

For instance, in the titration of an acetic acid solution it is desired to add standard NaOH solution until all the acetic acid is converted to sodium acetate. The hydrogen-ion concentration of sodium acetate solution is about $P_H^+ = 7·5$ to 9·5 so that phenolphthalein or another indicator exhibiting its colour change over this range of reaction is employed.

Again, in the Kjeldhal titration N/10 NaOH is added to a very dilute solution of sulphuric acid containing some ammonium sulphate. The "end point" is reached when all the sulphuric acid is converted to ammonium sulphate and sodium sulphate. Ammonium sulphate is faintly acid so that a trace of soda in excess will bring the mixture nearly neutral. Hence an indicator is chosen which is very sensitive at the neutral point—methyl orange, alizarin, rosolic acid have all been recommended. Such simple titration processes depend for their accuracy and reliability on a sudden change taking place in the reaction of the titrated fluid at some point during the addition of the alkaline solution, and the correct choice of an indicator which demonstrates this change. When dealing with very feebly dissociated acids and bases, of which proteins and some of their products of hydrolysis are excellent examples, such sudden changes of reaction do not occur. The titration process becomes more difficult. Moreover at any time it gives no information as to the rate of change of concentration of $H^{\cdot}$ ions taking place progressively step by step as the alkaline solution is added. The measurement of this is important, and its neglect is frequently the outcome of the fact that it is a laborious process. To do it colorimetrically would be tedious and, in small quantities of fluid, impossible. Electrometrically with an electrode vessel of the standard type and a tape it would be impracticable, since loss and contamination of the fluid by potassium chloride would inevitably occur. With a vertical electrode vessel filled by suction these measurements of change of $H^{\cdot}$ ion concentration during the titration process have been found quite practicable. There is no loss, practically no contamination by potassium chloride, and each determination of $H^{\cdot}$ ion concentration during the titration process takes about two minutes.

Apparatus. Fig. 2 is a scale drawing of the apparatus used for titrating 10 cc. quantities. A small beaker contains the fluid to be titrated and dipping into it is a small electrode vessel (*B*) of capacity 3 cc. and a capillary tube filled with saturated KCl solution, connected to the saturated KCl-calomel electrode. Passage of liquid bodily along the capillary is prevented by the insertion of a long tightly packed plug of cotton wool, and if necessary a clip on the rubber tube connecting it to the half-electrode. The conductivity of the potassium chloride is so good that readings may still be taken with the clip on and sometimes, if the tap on the half-electrode be not vaselined, with this tap shut. A standard burette is clamped above the beaker. Two wires from the potentiometer lead to the half element and the electrode vessel respectively.

Technique. The electrode vessel is filled with hydrogen, and, while the

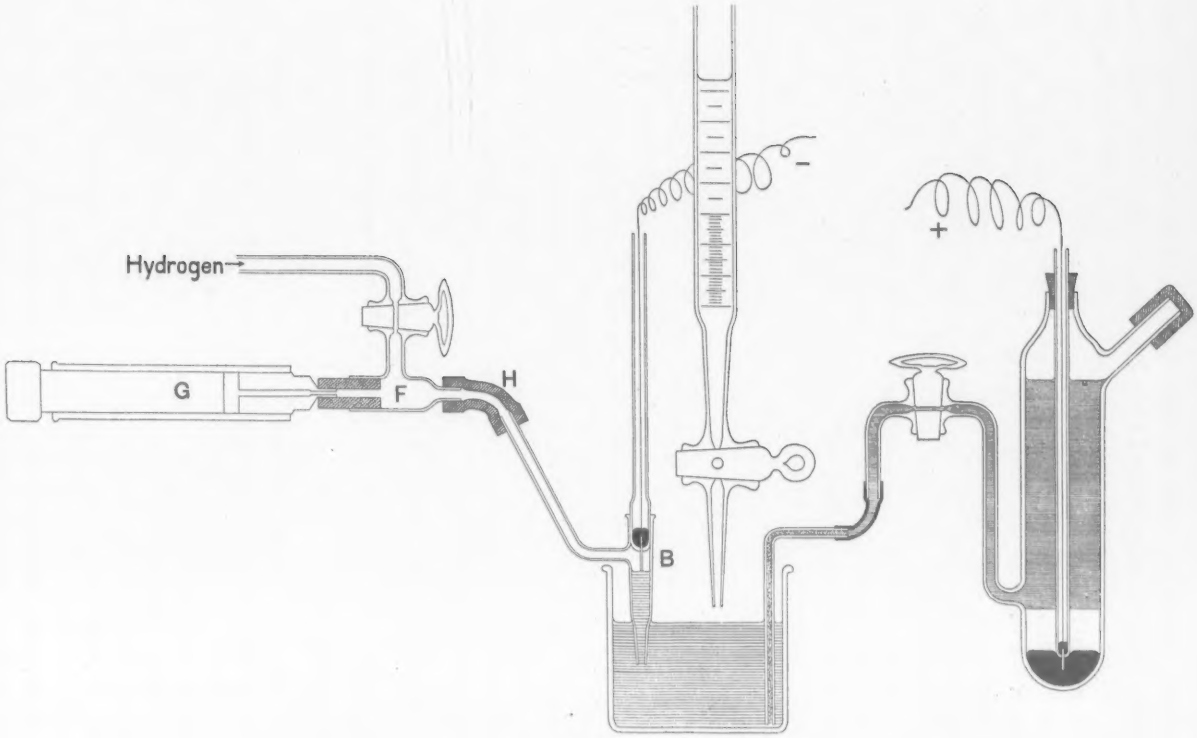

Fig. 2.

gas is still passing, its lower end brought below the surface of the measured quantity of liquid to be titrated which has been introduced into the beaker. The tap on the filling apparatus is turned off and by withdrawing the plunger, liquid is sucked up until contact is made. A reading of the potential indicates the reaction of the original liquid. The plunger is pushed home, a few drops of the titrating solution in the burette are run in, and by imparting a rotary motion to the beaker its contents are mixed until uniform throughout, and the reaction determined as before. In practice it is found best to draw the liquid up into the side tube and push it out several times in order to rinse the platinum point well between each reading.

From the results a curve may be plotted showing the relationship between the quantity of fluid added and the reaction. If any doubt exists as to the accuracy of the results obtained in this way, one or two points on the curve can be checked by single additions of N/10 alkali followed by determinations in a larger electrode vessel.

Example 1. 10 cc. of N/10 glycocoll solution containing in 1 litre 7·505 g. glycocoll and 5·85 g. sodium chloride [Sörensen, 1909, 1] were titrated with N/10 HCl electrometrically. Two separate titrations *A* and *B* were performed and subsequently the amount of potassium chloride accidentally introduced was found to be equivalent to 3·3 cc. of decinormal solution in one case and 3·0 cc. in the other, though no clip was used and the tap was open. The 10 determinations in titration *B* took 13 minutes; the 21 determinations in titration *A* took 43 minutes, though no particular attempt was made at speed.

Electrometric titrations of 10 *cc. glycocoll solution with N*/10 *HCl.*

Connecting fluid—saturated KCl solution. Half-electrode—saturated KCl-calomel. No correction for diffusion potential.

Vol. N/10 HCl added	E. M. F. in titration *A*	E. M. F. in titration *B*	Calculated from Sörensen's results
1 cc.	0·446, 0·445, 0·445	0·443	0·446
2	0·426, 0·426, 0·426	0·425	0·426
3	0·413, 0·414, 0·414	0·412	0·413
4	0·404, 0·404	0·402	0·403
5	0·394, 0·394	0·393	0·394
6	0·387, 0·387	0·386	0·386
7	0·381	0·379	0·379
8	0·374	0·373	0·373
9	0·369	0·367	0·367
10	0·365, 0·365, 0·365	0·362	0·362

Example 2. Advantage was taken of the opportunity now afforded of following the changes of reaction during a Sörensen formaldehyde titration.

A 4 % solution of Witte peptone was employed and the titration performed with every care. First colorimetrically, compensating for the colour of the peptone solution by a special tintometer, and then electrometrically.

1. Sörensen titration of 4 % Witte peptone solution colorimetrically.

Stage (1). *The neutralisation of the peptone solution.* Neutral red solution was used as indicator, and as a standard of neutrality a mixture of N/15 phosphate solutions (34·4 cc. acid + 65·6 cc. alkaline). 10 cc. of this solution —its reaction does not change on dilution [Szili, 1904]—to which 1 cc. of neutral red solution was added, were placed in a cylindrical vessel with a flat bottom *D*. A similar vessel surmounting this contained 10 cc. of Witte peptone *B*. Two corresponding vessels, *A* and *C*, contained distilled water, and 10 cc. Witte peptone solution plus 1 cc. neutral red solution respectively. Looking down *AC* the tint appeared redder than that seen looking down *BD*, and successive additions of N/10 NaOH solution were made until 0·5 cc. had been added, and the two columns were seen to match. It was considered that the 10 cc. of Witte peptone solution were neutralised by the addition of 0·5 cc. N/10 NaOH. Unfortunately the colorimetric result is misleading here. The P_H^+ value is 7·66 and not 7·07. The errors in P_H^+ found by Sörensen [1909, 2] in similar determinations with 2 per cent. Witte peptone solution containing 0·1 N NaCl and correcting for the colour of the solution by 2 drops of Bismarck brown and 2 drops of helianthin II were 0·18 and 0·12 in the same direction in two cases. Rosolic acid gave a similar result.

Stage (2). *Titration in presence of formaldehyde.* The problem is to discover how much 0·1 N NaOH must be added to a mixture of 10 cc. of Witte peptone solution and 10 cc. of neutral 40 per cent. formaldehyde solution in order that the resulting mixture should have the reaction $P_H^+ = 8{\cdot}68$ (7 borate + 3 HCl); what further addition must be made to bring P_H^+ value to 8·91 (8 borate + 2 HCl); and what still further addition will bring the value to $P_H^+ = 9{\cdot}09$ (9 borate + 1 HCl).

Using the same apparatus the solutions were arranged as shown on p. 422.

Formaldehyde bleaches slightly the colour of Witte peptone so that its colour must be compensated for by a solution similarly bleached. 1 cc. saturated sodium chloride solution is added to each formaldehyde-peptone mixture to inhibit the formation of polymethylimino-compounds. The same quantity was added to the 20 cc. of Sörensen mixture in *D* in order that the "neutral salt effect" on the phenolphthalein should be of the same order in

A.

10 cc. Witte peptone solution.
10 cc. formaldehyde containing 1·0 cc. of 0·1 % phenolphthalein in 50 % alcohol.
1 cc. saturated NaCl solution.
(4·8 cc. 0·1 N NaOH were added to this tube to make a match.)

B.

10 cc. Witte peptone solution.
10 cc. formaldehyde solution.
1 cc. saturated NaCl solution.

C.

20 cc. distilled water.

D.

20 cc. Sörensen mixture.
(7 borate + 3 HCl.)
1·0 cc. of 0·1 % phenolphthalein in 50 % alcohol.
1 cc. saturated NaCl solution.

A and *D*. The amount of 0·1 N NaOH added to *A* before a match resulted was 4·8 cc. *D* was now removed and replaced by *D'* containing 20 cc. (8 borate + 2 HCl) mixture, 1·0 cc. of 0·1 % phenolphthalein in 50 % alcohol, and 1 cc. saturated sodium chloride solution. A further 0·3 cc. of 0·1 N NaOH was required in *A* to make a match. *D'* was now replaced by *D''* containing 20 cc. (9 borate + 1 HCl) mixture, 1·0 cc. of 0·1 % phenolphthalein in 50 % alcohol, 1 cc. saturated NaCl. A further 0·3 cc. of 0·1 N NaOH was required to make a match. Hence by a colorimetric method it has been found that the following mixtures have the corresponding $H^{\cdot}$ ion concentrations given in the table:

P_H^+	Composition of mixture
7·07	10 cc. Witte peptone solution + 0·5 cc. 0·1 N NaOH
8·68	" " " + 10 cc. neutral 40 % formaldehyde sol. + 4·8 0·1 N NaOH
8·91	" " " + " " " + 5·1 "
9·09	" " " + " " " + 5·4 "

By a simple artifice the formation of polymethylimino-compounds in the above titrations may be prevented without the addition of sodium chloride solution. If, before adding the formaldehyde to the peptone solution some 0·1 N NaOH be added, then no cloudiness or precipitate forms. Repeating the above titrations in this manner and adding the solutions in the order named, the following results were obtained.

P_H^+	
8·68	10 cc. Witte peptone soln. + 4·0 cc. 0·1 N NaOH + 10 cc. neutral 40 % formaldehyde + 0·80 cc. 0·1 N NaOH
8·91	" " " + " " + 10 cc. neutral 40 % formaldehyde + 1·10 cc. 0·1 N NaOH
9·09	" " " + " " + 10 cc. neutral 40 % formaldehyde + 1·40 cc. 0·1 N NaOH

In Fig. 3, the relation between the compositions of these mixtures and their P_H^+ values is represented diagrammatically.

The points obtained using phenolphthalein are marked +. The neutral red point is marked #.

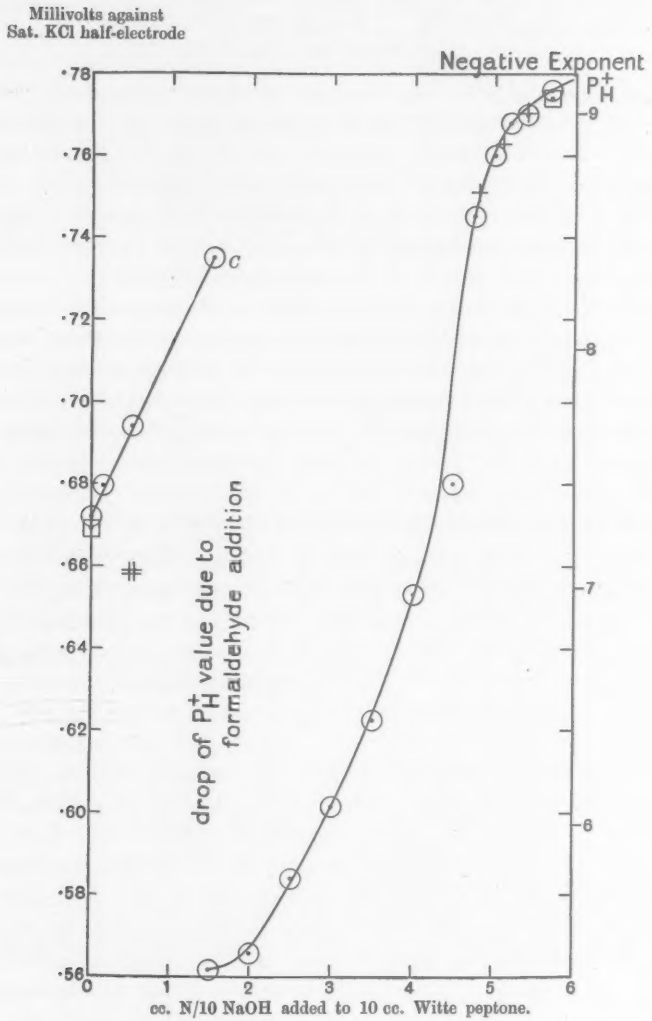

Fig. 3. Sörensen formaldehyde titration of 4 % Witte peptone performed electrometrically.

Note.—At *C* 10 cc. neutral formalin was added.

⊙ are points obtained in the titration process—electrode containing 0·3 cc.

□ are points obtained with large electrodes in separate determinations.

\+ are points obtained colorimetrically using phenolphthalein.

is point obtained colorimetrically using neutral red.

2. Sörensen titration of 4 % Witte peptone solution electrometrically.

10 cc. of peptone solution were taken and the same small electrode vessel as before. Connecting fluid and fluid in calomel half-electrode were saturated KCl solution; room temperature about 17°. As the alkaline formaldehyde solution becomes less alkaline on prolonged exposure to the air it is necessary to work with reasonable rapidity. After the addition of 1·5 cc. of 0·1 N NaOH solution the potential reading was found to be 0·7350; 10 cc. of neutral formaldehyde were then added and the potential immediately fell to 0·5615. Further additions of decinormal alkali were made—and corresponding potential readings taken—until after adding 4·5 cc. in all, the change of potential was so great, on account of the addition of the last 0·5 cc., that the ordinary rinsing out of the apparatus between two determinations was evidently not sufficient for this case, and the value 0·6800 is probably too low, the point being off the curve.

Experiments with solutions containing dissolved carbon dioxide.

The difficulties of $H^{\cdot}$ determinations of fluids containing carbon dioxide have been so thoroughly dealt with by Michaelis and Rona [1909], Hasselbalch [1910], and Michaelis and Davidhoff [1912] that their repetition here is unnecessary. It is sufficient to remark that before a final unchanging potential reading can be obtained the hydrogen atmosphere, the fluid, and the platinum electrode must all be in equilibrium, no further gas exchange taking place between them. This refers to hydrogen, carbon dioxide, and oxygen. If the hydrogen be not at 760 mm. a correction must be applied to the formula expressing the relation between the potential of the half element and the hydrogen ion concentration of the fluid.

Generally speaking, electrode vessels may be divided into two classes, those in which hydrogen is passed in turn through a small portion of the fluid examined, and then through a portion of the same fluid in the cell itself until equilibrium is attained, and those in which one portion of hydrogen only is used. The methods are referred to respectively as those of the "moving" and "still" hydrogen atmosphere.

In electrode vessels in which a "still" atmosphere is employed it may be considered that there are three degrees of accuracy with which the $H^{\cdot}$ concentration of a fluid containing carbon dioxide may be determined.

Firstly, the bubble of hydrogen may be brought to the surface of the fluid examined, the blackened platinum point saturated with hydrogen

adjusted so that it is just in contact with this surface, and the potential measured. The form of electrode used may be the V-electrode or the vertical electrode described. The potential will not represent accurately the reaction of the fluid. Owing to the diffusion of carbon dioxide from the fluid into the hydrogen the surface layers will rapidly become more alkaline than the solution was originally. The potential reading will therefore be high, falling slowly as equilibrium is established and only reaching a constant value some hours afterwards when that end has been attained.

Secondly, the small bubble of hydrogen may be passed backwards and forwards through the fluid in the cell a few hundred times before a reading is taken. By this means equilibrium is established, and it will be found that the value is constant and nearly correct. The electrode used may be a V-electrode manipulated in accordance with the instructions given by Michaelis [1912], or the vertical type filled by suction, or the Hasselbalch electrode. It will be seen that, though equilibrium is established between the hydrogen atmosphere and the fluid, the fluid has given up some carbon dioxide to the hydrogen and has therefore become more alkaline than it was originally and it is not the $H^{\cdot}$ ion concentration of the original solution that has been measured.

This brings us to the third step where after equilibrium is reached the hydrogen bubble is retained, but the fluid in the cell replaced by a fresh volume with which the hydrogen is again brought into equilibrium. This process may of course be repeated until a definite final potential reading is observed which will then represent the true reaction of the fluid containing carbon dioxide.

The only electrode vessel described which permits of this is the Hasselbalch electrode vessel. Michaelis used it to control the results obtained by him using the V-electrode and passing the bubble up and down many times to obtain equilibrium—the second case above.

Since finding that the same thing can be done quite simply with the same electrode vessel as that described for gas-free solutions, I have made a number of determinations of the reactions of carbonate solutions by its means and have obtained consistent results.

Technique. If the electrode vessel be tipped sideways when filling, the liquid drawn up may be made to enter the side capillary leaving a bubble of hydrogen. After closing the tap the apparatus may be taken in the hand and by a slight movement at the wrist the bubble made to pass from one end of the vessel to the other as many times as are necessary to obtain equilibrium.

Bringing the vessel into a vertical position again the lower end is dipped below the surface of the fluid in the beaker, and the glass tube moved up or down through the rubber stopper until the platinum point just touches the surface of the column of fluid standing up in the electrode vessel. This may now be wiped dry externally, placed in the trough, and a reading of the potential taken. The value obtained corresponds exactly to that obtained by the V-tube used in the manner advocated by Michaelis. When small quantities only of carbon dioxide are present, this value will be very nearly correct. It may be checked by attaching a rubber tube to the tube at *D* (Fig. 1), introducing the lower end of the vessel in a sloping position into the beaker again, aspirating at *D*, and opening the tap gently. In this way fresh solution is drawn into *B* in the place of the old solution which passes out through *D* without disturbing the hydrogen bubble. This fresh quantity of solution is now brought into equilibrium with the bubble of hydrogen as before. The process may be repeated indefinitely and the result obtained is of the third order of accuracy—previously only obtained by the Hasselbalch electrode. The pattern of electrode vessel having a ground glass joint cannot be used for these operations as the height of the platinum point in the vessel is not then adjustable. It is essential that the platinum point shall only just touch the surface of the fluid when a reading is to be taken, otherwise equilibrium between the electrode and the solution, instead of taking only a minute or two, may take hours. In these cases, where the investigated liquid contains carbon dioxide, with experiments lasting over a number of hours, the rubber joint has a further disadvantage over and above that already mentioned. Carbon dioxide, like hydrogen, permeates rubber, and though the rubber joint is immersed in potassium chloride solution, transpiration of gases through the rubber is not prevented, and this slow transpiration is detrimental to accurate work when experiments last several hours.

Carbonate solutions. In order to check the results obtained when using the vertical electrode filled by suction for fluids containing carbon dioxide, I have determined the hydrogen ion concentration of mixtures of a sodium carbonate solution and dilute hydrochloric acid. For each determination 12·5 cc. of 0·2 N sodium carbonate were taken and diluted to nearly 100 cc. Then a measured quantity of 0·1 N hydrochloric acid was added and the volume made to 100 cc. exactly. No barometric correction has been applied to the determinations for diminished hydrogen pressure due to the carbon dioxide present. Neither have any steps been taken beyond the use of saturated potassium chloride as connecting solution to correct for diffusion potential. The results are plotted on a curve (Fig. 4). Abscissae represent

cc. of 0·1 N HCl taken: while ordinates are proportionate to the P_H^+ values less a constant. It will be seen that a mixture of 12·5 cc. of 0·2 N sodium carbonate + 12·5 cc. 0·1 N HCl diluted to 100 cc. corresponds to a solution of 0·0125 molecular $NaHCO_3$ which is also 0·0125 N with respect to sodium chloride. At this point the potential is 0·7200 against the calomel saturated-KCl half-electrode corresponding to $P_H^+ = 8·13$. At this reaction phenolphthalein gives a pale pink colour, thus confirming the propriety of the analytical device of titrating carbonates in the presence of caustic alkali using phenolphthalein and methyl orange.

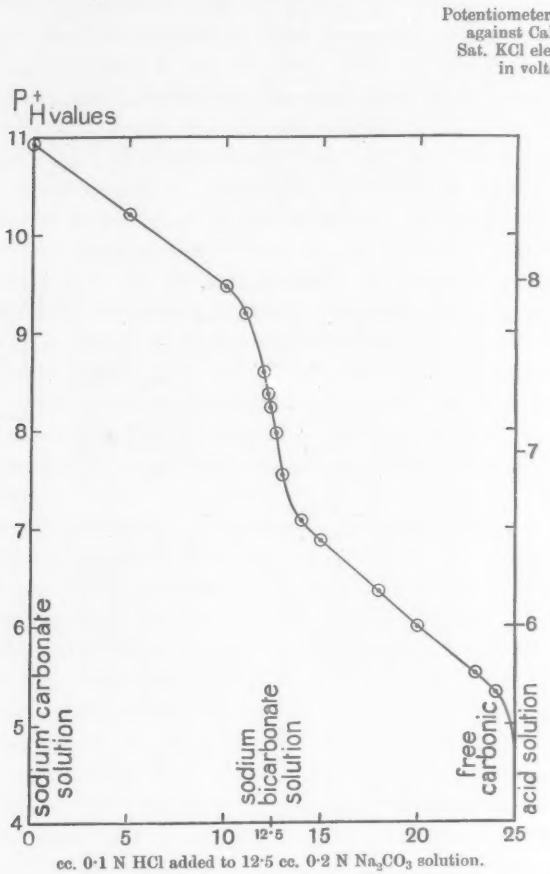

Fig. 4.

Mixtures of 12·5 *cc.* 0·2 *N* Na_2CO_3 *and varying quantities of* 0·1 *N HCl. diluted to* 250 cc.

Connecting fluid sat. KCl. Half-electrode calomel-sat. KCl.

Vol. of 0·1 N HCl	Potential π	$P_H^+ = \frac{\pi - 0{\cdot}251}{0{\cdot}0577}$
0	0·883	10·95
5	0·841	10·22
10	0·798	9·48
11	0·782	9·20
12	0·755	8·73
12·3	0·738	8·44
12·5	0·720	8·13
12·7	0·701	7·80
13	0·684	7·50
14	0·652	6·95
15	0·638	6·71
18	0·618	6·36
20	0·598	6·01
23	0·570	5·53
24	0·558	5·32

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XLII. SOME ESTERS OF PALMITIC ACID.

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The physiological importance and commercial value of the fats early attracted chemists to the study of their properties and composition. The interest which they then aroused has been fully maintained with the result that the present extensive literature on the compounds of the higher fatty acids deals almost entirely with the esters of glycerol to the exclusion of those of other polyhydric alcohols.

The chemistry of the fats may be said to have had its birth in the work of Chevreul [1823] to whose researches in the first quarter of the nineteenth century we owe the proof that the fats occurring in the animal body are triglycerides of higher fatty acids chiefly of stearic, palmitic and oleic. About forty years later Berthelot [1860] first succeeded in synthesising the mono-, di-, and tri-glycerides of many fatty acids and in identifying the tri-olein, tri-palmitin and tri-stearin so obtained with naturally occurring animal fats.

The method of synthesis adopted by Berthelot consisted in heating together glycerol and the fatty acid in a sealed tube to 200°–250°. This direct method of synthesis with various slight modifications has been largely used by later workers and still enjoys a wider application than the yields which are so obtained appear to warrant. Modifications of this method are chiefly directed towards eliminating the water formed during the reaction and thus enabling the process to be carried out at a lower temperature and also increasing the yield. A modification of this kind was introduced by Scheij [1899] and extended by Belucci and Manzetti [1911]. These workers found that the yields obtained by Berthelot's method could be greatly increased by carrying out the reaction under diminished pressure; thus Belucci and Manzetti synthesised triolein by heating together glycerol and oleic acid to 260° under a pressure of 20 mm., and state that in this way a yield of 95–98 %

was obtained; they afterwards found that the reaction could be carried out equally well under atmospheric pressure if a current of carbon dioxide were passed through the flask. Grün [1905] carried out the synthesis of diglycerides by warming the fatty acid and glycerol in concentrated sulphuric acid; thus *α*-*α*-distearin was obtained from glycerol and stearic acid, glycerol-disulphonic acid being formed as an intermediate product. None but primary alcohol groups are esterified in this reaction. Bloor [1910, 1912] applied this method to the condensation of mannitol with stearic and lauric acids; he identified his products as mannitol distearate and mannitol dilaurate respectively.

The halogen derivatives of glycerol have also been used in the preparation of glycerides. Partheil and Von Velsen [1900] prepared trilaurin and tripalmitin by heating the silver salts of these acids and tribromohydrin to 140° to 150° in the presence of xylene. A similar method was used by Guth [1903]; he prepared monostearin by heating together sodium stearate and monochlorohydrin, also *α*-distearin from *α*-dichlorohydrin, and *β*-distearin from *β*-dibromohydrin; tristearin was obtained from tribromohydrin and excess of sodium stearate. The yields obtained by these last two methods are not given.

Since the esters of the lower fatty acids are so readily prepared by the action of the acid chloride on the alcohol it seemed probable that this method might also be applied to the synthesis of esters of the higher fatty acids, not only with glycerol but also with other polyhydric alcohols. This method has been used recently by Grün and Schreyer [1912] who prepared *α*-myristo-*α*-chlorohydrin by the action of myristyl chloride on *α*-monochlorohydrin, and *β*-myristo-*α*-*α*-dichlorohydrin from myristyl chloride on *α*-*α*-diehlorohydrin. This is the only example of the use of this method which the writer has been able to find, though the wide use of the method in other fields makes it seem possible that some cases have been overlooked.

In order to test the efficiency of the above method for the synthesis of fatty bodies, palmityl chloride has been exclusively used and was prepared by the method described by Marie [1896] for melissyl and cerotyl chlorides. Its action was tried on ethylene glycol, glycerol, mannitol and glucose. In the case of glycerol esterification was effected by heating the two substances together to 120°. The action however was slow and this method was abandoned in favour of allowing the substances to react in the presence of pyridine with chloroform as a solvent. The action then took place in the cold and was completed by gently warming on the water bath.

Experimental.

Preparation of palmityl chloride. 45 g. of palmitic acid were mixed with 40 g. of phosphorus pentachloride (calculated amount 38 g.) in a distillation flask and the action started by warming. When the action was complete the excess of phosphorus pentachloride and the phosphorus oxychloride were distilled in vacuo on a boiling water bath; when these substances were removed the temperature was raised; the palmityl chloride distilled over as a colourless oil at 198°–200° under a pressure of 15 mm. Yield obtained 37 g. (77 %).

Preparation of glycol dipalmitate. 1 g. of dry glycol and 5 g. of pyridine were added to 12·8 g. of palmityl chloride (4 g. in excess of that required for complete esterification); about 50 cc. of dry chloroform were then added to dissolve the solid compound of pyridine and palmityl chloride formed; the contents of the flask turned a deep orange colour. The flask was closed and allowed to stand for 24 hours, it was then gently warmed on the water bath and well shaken; the chloroform was evaporated off and dilute sulphuric acid added to liberate the pyridine from its combination with palmityl chloride; the insoluble ester and palmitic acid (from excess of reagent) were filtered off and washed free from sulphuric acid and pyridine on a hot water filter; they were then dissolved in hot absolute alcohol and the free palmitic acid neutralised with alcoholic potash; the solution was evaporated to dryness and the residue transferred to a Soxhlet apparatus and extracted with ether. The ester crystallised from the ethereal solution on cooling in clusters of fine needles.

Yield before recrystallisation 7·5 g. (94 %). The substance so obtained was brownish yellow in colour and was soluble in hot and cold alcohol and also in chloroform, ether, ethyl acetate and the usual solvents for fats. It was recrystallised from a mixture of chloroform and alcohol since it was found that by this method the crystals obtained were white whereas when other solvents were used the colouring matter was thrown down with the crystals. The crystals so obtained were deposited in rosettes of fine needles; after four recrystallisations the substance had a constant m.p. of 65° (corrected). It was dried to constant weight in vacuo over sulphuric acid; it still, however, contained traces of moisture, which were only finally removed by heating in a toluene bath to 106°. The substance was identified by the results of elementary analysis and by the percentage of palmitic acid estimated by Hehner's method. As these values calculated for glycol monopalmitate,

glycol dipalmitate and for palmitic acid approach somewhat nearly to each other, these figures are appended for purposes of comparison.

	% Hydrogen	% Carbon	% Palmitic acid
Calculated for glycol monopalmitate	12·00	72·00	85·3
,, ,, glycol dipalmitate	12·27	75·84	95·2
,, ,, palmitic acid	12·5	75·01	100

Analysis:

(1) 0·2195 g.; 0·2406 g. H_2O; 0·6108 g. CO_2.
(2) 0·2598 g.; 0·2913 g. H_2O; 0·7227 g. CO_2.
(3) 1·8598 g.; 1·7750 g. palmitic acid.

	% Hydrogen	% Carbon	% Palmitic acid
Analysis (1)	12·44	75·9	—
,, (2)	12·45	75·84	—
,, (3)	—	—	95·5

The compound may therefore be identified as glycol dipalmitate corresponding to the formula

$$\begin{array}{l} CH_2OOCC_{15}H_{31} \\ | \\ CH_2OOCC_{15}H_{31}. \end{array}$$

Glycerol tripalmitate (tripalmitin). 1·2 g. of glycerol and 9 g. of pyridine were added to 15·8 g. of palmityl chloride (4·7 g. in excess of that required for complete esterification); 30 cc. of chloroform were added. The procedure followed was the same as that described for the glycol compound. In this case the ester obtained from the ether extract was brown in colour and much difficulty was experienced in obtaining white crystals. After two recrystallisations from chloroform and alcohol followed by four from ether the substance, though melting sharply at 62°, was still biscuit coloured. It was then dissolved in wet ether and boiled for six hours with charcoal. The substance which crystallised from the ether after filtration was quite white and was deposited in fine needles; m.p. 62° (corrected). It was sparingly soluble in hot alcohol and soluble in ether, chloroform and all the usual solvents for fats. Traces of charcoal clung to the substance with great obstinacy and could not be removed by the filtration of the ethereal solution. They were finally removed by filtering the substance itself in the steam oven through a very small filter. The substance was dried at 110°, and identified by the results of elementary analysis; the corresponding values calculated for di- and tripalmitin and for palmitic acid are appended for comparison.

	% Hydrogen	% Carbon
Calculated for dipalmitin	11·90	74·06
,, ,, tripalmitin	11·91	75·95
,, ,, palmitic acid	12·5	75·01

Analysis: (1) 0·2055 g.; 0·2222 g. H_2O; 0·5728 g. CO_2.
(2) 0·1945 g.; 0·2155 g. H_2O; 0·5405 g. CO_2.

	% Hydrogen	% Carbon
Analysis (1)	12·01	76·00
,, (2)	12·30	75·78

The substance was therefore identified as tripalmitin.

Owing to the difficulty experienced in purification sufficient substance was not available for a determination of palmitic acid.

Yield before recrystallisation 5·5 g. (50 %).

Yield of pure substance 2 g.

Mannitol hexapalmitate. 1·5 g. of mannitol and 8 g. of pyridine were added to 19·2 g. of palmityl chloride (4·8 g. in excess of that required for complete esterification). The procedure followed was identical with that described for glycol. The total yield of ester obtained after extraction in the Soxhlet apparatus and before further purification was 11·2 g. (78 %). The substance obtained was almost insoluble in cold alcohol, and very sparingly soluble in hot alcohol; it was easily soluble in ether, light petroleum, chloroform and ethyl acetate. It crystallised readily from a mixture of chloroform and alcohol or from ethyl acetate in rosettes of fine needles; m.p. 64·5 (corrected). It was dried at 106°.

The substance was identified by the results of elementary analysis and by the percentage of palmitic acid estimated by Hehner's method; these values calculated for mannitol hexapalmitate, mannitol pentapalmitate and for palmitic acid are also appended.

	% Hydrogen	% Carbon	% Palmitic acid
Calculated for mannitol hexapalmitate	12·05	76·03	95·4
,, ,, mannitol pentapalmitate	11·90	75·26	93·37
,, ,, palmitic acid	12·5	75·01	100

Analysis: (1) 0·2245 g.; 0·2430 g. H_2O; 0·6270 g. CO_2.
(2) 0·2135 g.; 0·2335 g. H_2O; 0·5940 g. CO_2.
(3) 1·7067 g.; 1·6220 g. of palmitic acid.

	% Hydrogen	% Carbon	% Palmitic acid
Analysis (1)	12·03	76·19	—
,, (2)	12·14	75·90	—
,, (3)	—	—	95·06

The substance was therefore identified as mannitol hexapalmitate corresponding to the formula

$$C_{15}H_{31}CO.O.CH_2.(CH.OOCC_{15}H_{31})_4.CH_2.O.OCC_{15}H_{31}.$$

Glucose pentapalmitate. 1 g. of glucose and 9 g. of pyridine were added to 8·3 g. of palmityl chloride (2·4 g. in excess of that required for complete esterification). The procedure followed was the same as that previously described; as in the case of the glycerol compound decolorisation with charcoal and subsequent filtration of the melted ester were necessary. The total yield obtained after extraction in the Soxhlet apparatus was about 5 g. (83 %). The total yield of purified material was 2·8 g.

The substance obtained was very slightly soluble in cold alcohol and only sparingly soluble in hot alcohol; it dissolved readily in all the solvents commonly used for fats. It was recrystallised from ethyl acetate and melted at 62° (corrected). The substance was readily hydrolysed by alcoholic potash as was shown by the rapid darkening of the solution due to the action of the alkali on glucose, coupled with the fact that the substance was soon completely dissolved. The action of the glucose on the alkali prevented a saponification value from being taken. An attempt was made to estimate the palmitic acid by Hehner's method; a known weight of the substance was saponified with alcoholic potash, the alcohol evaporated off and the soap dissolved in water. The solution was very dark owing to the presence of the resins already mentioned, and it was found on acidifying and attempting to filter off the palmitic acid that the presence of these substances caused the palmitic acid to pass through the filter paper. An attempt to effect the complete acid hydrolysis and afterwards to estimate the glucose was next made; 0·397 g. of the ester was placed in a flask with 50 cc. of absolute alcohol in which, even on boiling, it only partially dissolved, the greater part sinking as oily drops; 25 cc. of 20 % hydrochloric acid were added and the flask boiled under a reflux condenser for 84 hours. At the end of this time the hydrolysis was still incomplete as was shown by the presence of small drops of insoluble ester in the boiling alcohol. When the contents of the flask were filtered to remove the unchanged ester and the filtrate neutralised the presence of glucose was shown by the reduction of Fehling's solution. Owing to the impossibility of removing the unchanged ester quantitatively the glucose could not be estimated. It was therefore necessary to rely solely on the combustion results for the identification of the substance.

0·2233 g.; 0·2410 g. H_2O; 0·6163 g. CO_2.

	% Hydrogen	% Carbon
Analysis	11·99	75·26
Calculated for glucose pentapalmitate	11·82	75·32
" " " tetrapalmitate	11·66	74·20

The substance was therefore identified as glucose pentapalmitate corresponding to the formula

$$CH_2 . OOCC_{15}H_{31} . (CH . O . OC . C_{15}H_{31})_4 . CHO.$$

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